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EFFECT OF AGE UPON DARK ADAPTATION

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It is a matter of common observation that older people are less well able to 'see in the dark' than younger people. Nevertheless, some workers who have drawn up standards of normality of dark adaptation, in order, for example, to detect deficiency of vitamin A, have failed to allow for this [e.g. Jeans, Blanchard & Satterthwaite, 1941]. Moreover, other workers have definitely stated that dark adaptation is unaffected by age [Korb, 1939]. Stewart [1941], however, found an appreciable deterioration with age. These discrepancies in the literature may in part be due to differences in the technique of measuring dark adaptation. Jeans and his colleagues and also Korb measured the early part of the process, that is, the adaptation mainly of the cones, whilst the technique adopted by Stewart measured almost entirely the adaptation of the rods. It might then appear that cone adaptation deteriorates with age but that rod adaptation is unaffected. Yet Hecht & Mandelbaum [1939] have found very little change in rod threshold with increasing age but an appreciable rise in cone threshold [Mandelbaum, 1941].

It is clear then that the question of variation of dark adaptation with age is by no means settled. Moreover, if dark adaptation does in fact deteriorate with age, two further questions arise: first, is the extent of the deterioration sufficient to make it necessary to allow for this in setting up standards of normality; and second, what is the cause of the deterioration?

During the past two years, we have measured the dark adaptation (final rod threshold) in over two thousand individuals between the ages of 10 and 70 and we believe that our results go some way towards answering these questions. We have found a progressive lowering of the power of dark adaptation with advancing years, we have determined its extent and we have been able to find a likely explanation of the phenomenon.

Experimental

Material

Since the various groups we have studied show slight but definite difference in the average value of dark adaptation [Robertson & Yudkin, 1944] we shall, in this paper, confine our attention to the results obtained with a single group of be compared, for example, with the correlation coefficient of 0·46 for the physical resemblance between children and their parents.

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* Sir Halley Stewart Research Fellow.

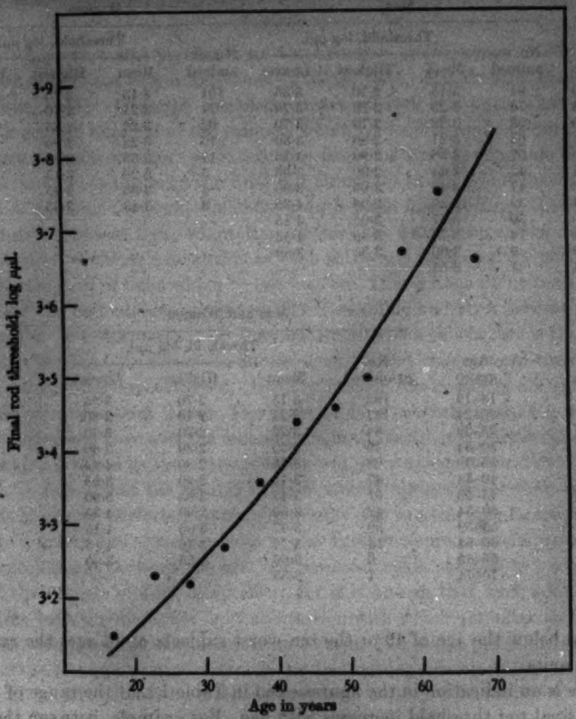

Fig. 1. Variation of dark adaptation with age.

Discussion

Cause of deterioration of dark adaptation with age

Booher & Williams [1938] suggested that the poorer performance of older subjects with the bio-photometer might be due to their diminished visual acuity. With their apparatus visual acuity affects performance, since the test involves perception of small spots of light. With the apparatus which we have used visual acuity plays a very small part; the test object in our apparatus is large, subtending an angle of 6° at the eye, and moreover the subject is encouraged to look for the presence or absence of light rather than the outline of the test object. Decreased visual acuity is therefore unlikely to be the cause of the deterioration of performance with age which occurs with our apparatus.

Other workers have suggested other reasons for the poorer dark adaptation of older people and these have been summarized by Ferree, Rand & Lewis [1935]. Although they were studying a somewhat different problem, namely the effect of low illumination on visual acuity, the factors which they discuss include those which might apply to the deterioration of dark adaptation. They state: 'Among the reasons why an old eye needs more light, and for that reason derives more benefit from an increase in the amount of light, the following five may be mentioned: its smaller pupil, the inferior imaging power of its refracting media, the diminished transparency of the media, the decay in all its processes of adaptation and adjustment and the failing powers of the retina itself.'

Age, pupil size and dark adaptation

Of the five factors mentioned by Ferree and his collaborators the first can perhaps most readily be measured and its possible effect on dark adaptation quantitatively studied. Let us for a moment assume that the other factors such as sensitivity of the retina and transparency of the ocular media do not change with advancing years. Then, as the pupil diminishes in size with age, and thus the amount of light entering the eye decreases, it would be necessary to increase the amount of external illumination proportionately in order to produce the same effect on the retina. If the pupil at 40 years has, let us say, an area half that of the pupil at 20 years, the amount of external light necessary to produce minimal stimulation after complete dark-adaptation will be twice as much at 40 years as at 20 years. If then we know the average size of the dark-adapted pupil at different ages we can calculate the relative amount of external light which will be just perceptible by a dark-adapted eye of different ages; given this threshold for any one age, we can thus calculate the threshold for different ages. (The possible bearing of the Stiles-Crawford phenomenon on these considerations will be discussed below.)

Although we have not been able to measure the actual size of the pupils of our subjects, data on the average size of the dark-adapted pupils at various ages have been published [Nitsche & Günther, 1930]. These values are shown

TABLE 2. Variation of amount of light reaching retina due to change in pupil size with age

Age	Radius of pupil mm. [from Nitsche & Günther, 1930]	Area of pupil mm.^{2}	Proportion of incident light reaching retina*
20	4·0	50·2	178
30	3·5	38·3	136
40	3·0	28·2	100
50	2·5	19·6	69·5
60	2·05	13·2	46·8
70	1·6	8·05	28·1

* Proportion of incident light reaching retina at age of 40 has been given the arbitrary value of 100.

in Table 2, wherein are also shown the relative amounts of incident light that would reach the retina at different ages from a constant source. If at 40 years this amount is given the arbitrary value of 100, at 20 years it would be 178 and at 60 years 46·8 (Table 2, column 4). From Fig. 1 the value of the threshold at

Table 3

	'Expected' threshold		'Observed' threshold
Age	μμl.	log μμl.	log μμl. (from Fig. 1)
20	1350	3·13	3·16
30	1840	3·26	3·26
40	2400	3·38	3·38
50	3460	3·54	3·50
60	5130	3·71	3·65
70	8750	3·94	3·81

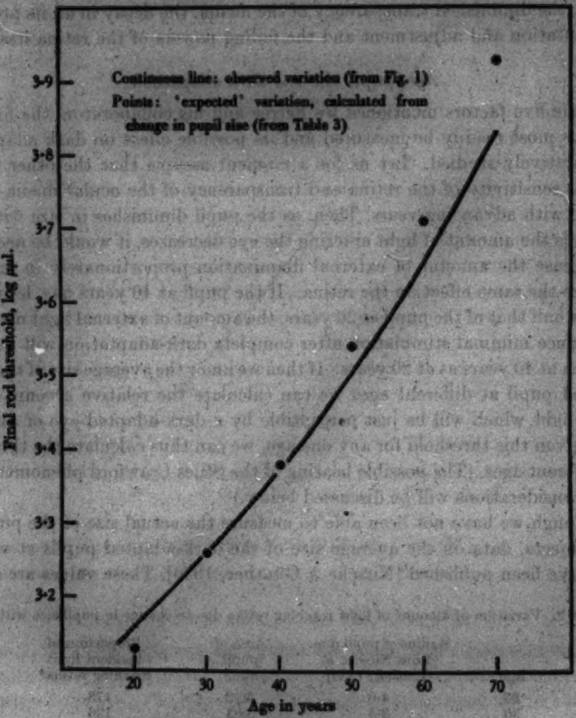

Fig. 2. 'Expected' variation of dark adaptation with age due to diminution of pupil size with age.

40 years is 2400 $\mu\mu$l. or 3·38 log $\mu\mu$l. From this value and the relative values given in Table 2 we may now calculate the expected thresholds at other ages (Table 3). When these values are compared with the actual values obtained by us, the agreement is found to be very close (Fig. 2).

It seems legitimate therefore to conclude that the deterioration of dark adaptation with age can be explained entirely by the decrease in the size of the pupil with age. It is not necessary to assume either a decreased sensitivity of the retina or a diminished transparency of the ocular media or any other supposed or possible change in order to explain the phenomenon. It should however be emphasized that our discussion has been concerned with the average dark adaptation in groups of individuals. The effect of pupil size on determining the dark adaptation in a single individual can only be assessed by direct measurement of the pupil. Such measurements would also make it possible to discover the extent to which the range of variation in dark adaptation in a given age group is due to variations in pupil size.

The Stiles-Crawford effect

Stiles & Crawford [1933] showed that the luminous efficiency of the periphery of the pupil is less than that of the central portion so that the visual effect of light with increased size of pupil does not increase as rapidly as the area of the pupil. If this were true in all conditions, we should expect that the smaller pupil of older subjects would be proportionately more efficient than the larger pupil of younger subjects; the threshold would then not increase as rapidly with age as we have calculated from the decrease in area. A more detailed consideration of the reported work on the Stiles-Crawford effect, however, shows that it is legitimate to assume a strict proportionality between pupil area and apparent brightness in the conditions which obtained in our experiments. The original observations of Stiles and Crawford and the confirmatory work of Dziobek [1934] and Wright & Nelson [1936] were all carried out with a fairly high field brightness; later work suggests that, for conditions approaching complete dark adaptation, the effect disappears. Crawford [1937] showed that the light passing through the periphery has less apparent brightness only if the field brightness is high or if it is the fovea which is being stimulated. In conditions of low brightness and parafoveal stimulation the luminous efficiency of the light is independent of the part of the pupil at which it enters. Sloan [1940] has followed this by an assessment of the effect of pupil size on the threshold of the dark-adapted eye. She finds that the product, threshold × size of pupil, is sensibly constant so that the visible effect of threshold brightness is directly proportional to the area of the pupil. Our calculations of the effect of the diminishing size of pupil with age on the threshold of the dark-adapted eye are therefore justified.

Standards of normality

Since the normal range of dark adaptation is fairly wide, the comparison of individual values with any fixed 'standards of normality' is not very satisfactory. Such standards might be of some use, however, in assessing the general status of dark adaptation in a large group, the distribution of the values being compared with the distribution obtained from a supposedly normal group. But even so our results indicate that, in such comparisons, it is necessary to take into account the effect of age. We have adopted this method in comparing various groups of subjects and these results will be reported in a later communication.

SUMMARY

1. By measuring the final rod threshold of 758 factory workers between the ages of 14 and 71, it has been shown that there is a progressive deterioration of average dark adaptation with increasing age.

2. For an increase of 10 years in age, this deterioration ranges from about 0·10 log unit between the ages of 20 and 30 years to about 0·15 log unit between the ages of 50 and 60.

3. It is possible to explain this phenomenon quantitatively by the progressive decrease in the size of the pupil with advancing years.

4. The bearing of these findings on the question of setting up standards of normality of dark adaptation is discussed.

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THE ACTION OF VITAMIN D UPON THE INCISOR TEETH OF RATS CONSUMING DIETS WITH A HIGH OR LOW CA:P RATIO

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Vitamin D is one of the factors essential for the proper formation of teeth, and in its absence the same changes are seen that occur in lack of Ca and P. The constantly developing incisor tooth of the rat is a very convenient object for studying the effects of vitamin D upon both the teeth and the body generally, as any change in Ca or P metabolism is immediately reflected in the appearance of the dentin, Erdheim [1911] having aptly compared the rat's incisor with the drum of a kymograph.

The effects of massive doses of the vitamin upon the normal rat's incisor have been described by Schour & Ham [1934] and after parathyroidectomy by Schour, Tweedy, Chandler & Engel [1937]. In both instances, the dentin showed a hypocalcified stripe followed by a reactive hypercalcified zone. Downs [1932] described the effects of vitamin D upon the teeth of rats on diets with various Ca:P ratios. The vitamin was incorporated into the diets and thus the effect was prophylactic. He came to the rather unexpected conclusion that the vitamin acted deleteriously with diets with a lowered P content, causing the histological changes to be more marked, but with diets with a lowered Ca content the vitamin acted beneficially.

The reaction of the tooth to curative doses of vitamin D after the establishment of well-marked dietary rickets does not, however, appear to have been followed. In the present paper the effects of the vitamin upon the teeth of rats with rickets caused by diets with abnormally high or low Ca:P ratios are described. Preliminary reports of some of the findings have already been published [Irving, 1941, 1943].

Methods

Animals

Albino rats of the Wistar Institute strain were kept in a special animal house at a temperature of 68–70°. Males and females were used indiscriminately, the results found being the same in both sexes. After weaning at 25 days, the young rats were housed by litters in steel cages and fed on the standard stock diet used in this department. When the animals weighed between 50 and 60 g., which was about 7 days after weaning, they were transferred to one of the experimental diets for 28 days.

Diets of the experimental animals

Three experimental diets, of normal, high and low Ca:P ratio, were used. They were based upon the rachitogenic diet no. 2965 of Steenbock & Black [1925], the Ca and P contents being altered by the addition of various amounts of $CaCO_3$ (B.P.) and $Na_2HPO_4.2$ aq. (Merck *puriss.*). The basal diet, consisting of yellow maize meal 76 parts, wheat gluten 20 parts, and NaCl 1 part, contained 0·035 % Ca and 0·0206 % P. The following was the composition of the three diets used, the figures being checked by analysis:

Diet no.	Ca %	P %	Ca:P ratio
1	1·60	0·87	1·8
2	1·21	0·28	4·4
3	0·122	0·42	0·29

They were given slightly moistened with water. After 28 days on one or other of these diets the negative control rats were removed and placed in separate cages. When vitamin D was administered it was given in one dose by mouth, using a micrometer syringe, the vitamin being dissolved in nut oil.

Treatment of animals

Three different experimental procedures were applied to rats of each of the groups receiving the different diets. These procedures were:

(*a*) Two rats of a litter acted as controls and received no dosage. Three rats were injected with 2 % NaF solution to act as a marker. Three rats were injected with 2 % NaF solution and given 9·2 i.u. vitamin D. All rats were then continued on the experimental diet for a further 10 days before being examined. Morgareide & Finn [1940] have stated that F affects the response of rickets to vitamin D. This was not found in the present work.

(*b*) One rat of a litter acted as a control and the rest were given graded doses of vitamin D ranging from 1·15 to 30 i.u. They were continued on the experimental diet for another 10 days and then killed and examined.

(*c*) All rats were given 18·4 i.u. vitamin D and were then continued on the experimental diet, being killed successively at regular intervals thereafter.

Owing to the fairly high vitamin D content of the stock diet, it was necessary to keep the animals for 28 days on the high-ratio rachitogenic diet, not 21 days as is usual, since if the shorter period was adopted, a proper line response to the vitamin was not obtained. This occurrence, using stock diets with a too high vitamin D content, has been noted by McCollum, Simmonds, Shipley & Park [1922], Bourdillon, Bruce, Fischmann & Webster [1931], Coward [1938] and others. To ensure comparable results, the animals on the other diets were also given them for 28 days.

In addition, a number of animals which had been used for vitamin D assay were also examined. These were put, when 14 days of age, on a diet poorer in vitamin D than the standard stock diet; when they had attained 50–60 g. in weight, they were transferred to the high-ratio rachitogenic diet for 21 days, were then dosed and kept on the rachitogenic diet for a further 10 days, being then killed and examined. These animals gave results which were identical with those found in the other animals on the high-ratio diet. In all cases the animals were weighed, after being put on the experimental diets, twice weekly.

Examination of the rats

Histological. The upper incisor teeth were cut in longitudinal section by the methods previously described [Gaunt & Irving, 1940], save that propyl alcohol was used for the final dehydration and clearing of the tissues. The sections were cut at 12μ, and stained with haematoxylin and eosin. Schour & Ham [1934] have shown that haematoxylin-stained tissues in teeth are well calcified and eosin-stained structures poorly calcified. The labial side of the teeth was examined and the width of the reactive zones measured with a micrometer eyepiece. The changes on the lingual side were also noted.

Chemical. The ash content of the right femur, tibia and fibula was determined in a number of litters, by heating the dry, fat-free bones in an electric muffle at about 700° till they had attained constant weight.

Line test. The degree of rickets and of healing was determined in all rats by the line test applied to the lower end of the radius and ulna [Dyer, 1931; Coward, 1938]. This method groups the epiphyseal appearance into seven grades, 0 being florid rickets and 6 complete healing.

Sodium fluoride injections. In order to act as a marker on the tooth, in a number of cases NaF was injected subcutaneously at the same time that the animal received vitamin D.

Most work using NaF as a marker has been done with two injections, separated by an interval of time, and measuring the increment of dentin between the two dental responses. When only one injection was given, as in the present experiments, it was necessary, in order to interpret the result of the injection, to make a few preliminary experiments using normal rats. The result of NaF injection is to cause a hypocalcified zone followed by a hypercalcified stripe. According to Schour & Smith [1934], the hypocalcified layer represents the immediate response to the injection, but the present writer has found that the outer edge of the hypercalcified stripe corresponds in time more accurately with the injection, the hypocalcified stripe being predentin formed before the injection was given. This conclusion is partly based on measurements taken from the teeth of rats which were killed at 6-hourly intervals after the administration of NaF, and also on figures obtained from five stock rats which were injected and killed exactly 10 days later. In the latter experiment the average width of predentin and dentin to the outer edge of the hypocalcified stripe was 174μ, while the same width, but to the outer edge of the hypercalcified stripe, was 162μ. This latter figure gives a daily increment of 16·2μ, which agrees well with that of 15·99μ obtained with alizarin red by Schour & Hoffman [1937]. As a result of these findings, all measurements were taken from the outer side of the hypercalcified line. The results obtained from NaF injections will be published in more detail elsewhere.

The animals on diet 1 were given 0·2 c.c. 2 % NaF without ill effect and with a well-marked reaction in the tooth. Those on diet 2 were at first given the same dosage, but this was found to have no effect upon the tooth and the effective dose was found to be 0·4 c.c.; this dose is usually fatal for a rat of corresponding weight eating a diet of normal Ca:P ratio. When the animals on diet 3 were given 0·2 c.c. of the 2 % solution, all of them went into profound tetany, most dying within 3 hr. The dose level for these animals had to be reduced to 0·1 c.c., and even then was sometimes fatal. This variable susceptibility to the action of NaF is presumably bound up with the level of the blood Ca.

The NaF line was used to check the measurements of the reactive dentin due to vitamin D as follows: in the case of high-ratio diets, the response in the labial dentin consisted of a deeply calcified zone of new dentin, often laid down in stripes and always edged by predentin of normal width and appearance. Calculations from the NaF line showed that the length of time of action of vitamin D could be computed by counting the number of stripes in the new dentin, including the predentin as one stripe, each stripe corresponding to a day's increment; or by dividing the total width of new dentin plus predentin by the predentin width (Fig. 4). Typical figures illustrating these points are given in Table 1. In the case of low-ratio diets, the labial response was entirely different, as will be explained later, and the above method could not be applied, but the NaF line acted as a guide in interpreting the histological picture. On the lingual side the new dentin was often laid down in stripes

TABLE 1. Comparison of the different methods of computing the length of time of vitamin D action. All animals were on diet 2 and were given 9·2 i.u. vitamin D

Reactive dentin and predentin					
Predentin width (μ) (1)	Predentin + dentin width (μ) (2)	(2)÷(1)	No. of stripes	Width of dentin + predentin up to NaF line	Time of action of D calculated from NaF line days
14	80	5·7	6	130	6·2
16	138	8·6	8	164	8·4
12	40	3·3	3	106	3·8
16	80	5·0	5	170	5·1

which could be counted, but as is usual on this side of the tooth, the predentin was too indefinite to be measured and no clear NaF line was found.

The distance of the NaF line from the odontoblasts was also used in measuring the rate of labial incremental dentin growth during the last 10 days of the experiment.

RESULTS

Diet 1. Normal Ca:P ratio, 1·8. A small experiment was conducted on two litters of rats to see how effective the Steenbock & Black diet was for tooth calcification when the Ca and P levels were within normal limits, both with and without vitamin D.

In all rats, the growth rate was very slow while on this experimental diet, being about half that of stock rats of corresponding size; vitamin D dosage made no difference to the rate of growth. The line test in all rats varied between 5·5 and 6 and the average bone ash was 57·90 %. The average value obtained from stock animals of comparable weight was 51·68 % and from stock animals of the same age was 59·93 %. In other respects the animals appeared entirely normal.

One litter of rats was treated with procedure (*a*). The animals on the diet alone had teeth which were quite normal in appearance save that the predentin was rather wider than usual, on an average 24μ. The apposition rate, as calculated from the NaF line, was slower than normal, being only 124μ during the last 10 days, as against the 162μ found in stock rats. The teeth of those rats treated with vitamin D differed in some respects from these. The more recently formed dentin showed an abrupt increase in haematoxylin staining, indicating an improved calcification due to the vitamin. In other respects they were entirely normal, with an average predentin width of 15μ, and an apposition rate of 151μ during the 10 days after injection. From a comparison of the edge of the reactive zone with the NaF line, the vitamin was found to have acted for 9·5 days.

The other litter of six rats was treated with procedure (*b*), graded doses of vitamin D, ranging from 1·15 to 18·4 i.u., being given. In the negative control, the predentin was, as with those previously mentioned, wider than usual, being 21μ. In all the rest the predentin width was normal. In animals getting 1·15 and 2·3 i.u. the dentin was uniformly stained, but in those getting 4·6 and higher dosages a reactive zone of better calcified new dentin was found, similar to that just described (Fig. 5). On measurement, it was found that the vitamin had acted for about 8·9 days in each rat which showed this reactive dentin, a figure confirmed by the number of stripes in the new dentin plus the predentin, which was 9 in each case. In all teeth, the vitamin was acting as strongly when the animal was killed as when its action began. The changes on the lingual side of the tooth were qualitatively the same as those on the labial side.

Diet 2. High Ca:P ratio, 4·4. Twenty-one litters of rats, including some which had been used for vitamin D assay, were examined. The growth rate in all rats was extremely poor, as is usual with the Steenbock & Black diet, but in very few cases was weight lost, and only one rat of all those examined showed any spontaneous healing of rickets. The bone ash values in these rats were low, varying between 22·15 and 36·60 %. The administration of vitamin D had no effect upon these values. The line values in the dosed rats were very variable, as is usually found, and varied between 0 and 5.

Qualitatively, the same effect of vitamin dosage was found in the teeth of all the animals getting vitamin D. The negative control animals showed all the signs of vitamin D deficiency, with wide predentin and vascular inclusions (Fig. 6). The animals dosed with vitamin D had a stripe of reactive new dentin, staining deeply with haematoxylin and affecting only the matrix laid down after the vitamin had been given. This new dentin was bordered with predentin of normal width. The predentin laid down before this was either completely unchanged or else showed a few scattered calcified granules. This new calcification of dentin took from 24 to 48 hr. to reach its height but was continuing equally intensely when the animals were killed, no matter what was the dose of D. The new dentin was often laid down in distinct stripes which could be counted and so used, as explained above, in computing the time of action of the vitamin (Fig. 7). The intensity of the calcification was equally marked whether it started early or late. These changes were best seen in the intermediate third of the tooth, using procedures (*a*) or (*b*), since this part consisted largely of old dentin and predentin formed before the vitamin had been given. The proximal third was almost entirely new dentin formed during the last 10 days of the experiment after the vitamin had been given. The changes on the lingual side of the tooth were the same as those on the labial side.

Four litters were treated with procedure (*a*). The animals receiving no vitamin D had an incremental dentin growth of 116μ during the last 10 days of the experiment, and, calculating from the very wide predentin width, calcification had been delayed on an average 8·3 days, instead of the usual 24 hr. In one rat, no calcification had occurred at all in the predentin laid down at the time of injection, and no NaF line was seen. Administration of vitamin D increased the incremental rate to 134μ, a value still below normal. The vitamin, at this dose level, had acted, on an average, for 5·9 days, the values found ranging from 3 to 8 days. The fact that the vitamin had acted for only about half of the time after it was given may account for the low value of the dentin increment. No obvious correlation was seen between the time of action of the vitamin and the line test result.

The rats from fifteen litters were given graded doses of vitamin D as in procedure (*b*). In all cases, a response in the dentin, consisting of a deeply hypercalcified stripe, was found. The width of the stripe, or time of action of the vitamin, varied, when a large number of animals were used and the results averaged, with the strength of the dosage. The response of the dentin in a comparable group of rats is plotted against the logarithm of the dose in Fig. 1, and the results from the line test are also included. It will be seen that, as is usual, the response to the dose is logarithmic in type in each case.

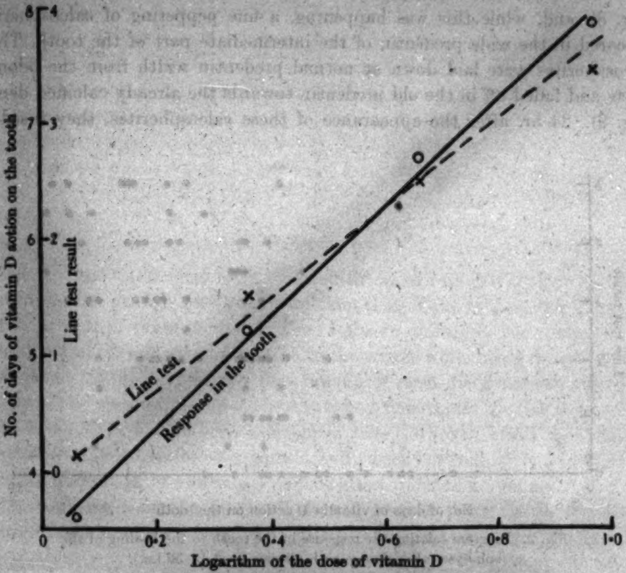

Fig. 1. Curve relating the response in the tooth and the healing of the epiphyses to the logarithm of the dose of vitamin D.

Comparison of the time of onset of the dentin response with the line test showed that the latter was a much less sensitive index of vitamin action. A diagram in which the two are compared is given in Fig. 2. This indicates that the vitamin must act in the body at least 4 days before any demonstrable change occurs in the epiphysis.

In order to study in more detail the early action of the vitamin, two litters of rats were dosed with 18·4 i.u. after the usual period on diet 2, and were thereafter killed at 24 hr. intervals (procedure (*c*)).

As is frequently found, the two litters differed in their response to the vitamin, though the responses of the individual rats of each litter were consistent among themselves. Thus one litter showed an epiphyseal response as indicated by the line test on the 5th and subsequent days, while the other did not show this till the 7th day.

The earliest response of the teeth to the vitamin was seen at the extreme proximal end of the tooth. This occurred as a small mass of calcospherites which appeared after the 1st day in one litter and after the 2nd day in the other one. These calcospherites fused within 48 hr. to form normal dentin (Fig. 8), and, while this was happening, a fine peppering of calcospherites appeared in the wide predentin of the intermediate part of the tooth. These calcospherites were laid down at normal predentin width from the odontoblasts and faded off in the old predentin towards the already calcified dentin (Fig. 9). 24 hr. after the appearance of these calcospherites, they fused to form deeply staining dentin, with normal predentin between it and the odontoblasts (Fig. 10). As this reactive dentin widened in animals killed later, it was possible to measure it and estimate the time of action of the vitamin in this part of the tooth. The lingual side of the tooth responded at the same time and in a similar manner by the formation of a narrow, deeply staining, stripe.

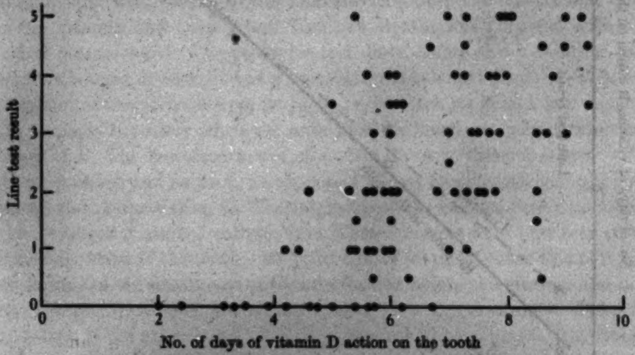

Fig. 2. Diagram relating the response in the tooth to the healing of the epiphyses after dosage with vitamin D (1·15–30 i.u.).

These results are summarized in Table 2. It will be apparent from the above description that the extreme proximal end of the tooth reacted at least 24 hr. before the more distal parts; this is also seen in the table; also that in the case of litter 220 the vitamin must have begun to act on the tooth almost immediately after it was given, as the predentin takes about 24 hr. to calcify. In confirmation of earlier observations, it will be seen that here also a positive line test did not occur till the vitamin had acted at least 4 days on all parts of the tooth.

TABLE 2. Changes in the teeth of rats on diet 2 killed daily after dosage with 18·4 i.u. vitamin D

Litter no.	Time killed after vitamin D dosage days	Line test	Reaction in proximal dentin	Time of action of D on intermediate dentin days
220	1	0	+	0
	2	0	+	0
	3	0	+	1
	4	0	+	3
	5	2	+	4
	6	2	+	5
	7	1·5	+	6
	8	1·5	+	6
221	1	0	0*	0
	2	0	+*	0
	3	0	+*	0
	4	0	+	1
	5	0	+	2
	6	0	+	3
	7	1·5	+	5

* See Fig. 8.

Diet 3. Low Ca:P ratio, 0·29. Eleven litters of rats getting this diet were examined. The growth rate was faster than that of the rats on diet 2, but not so fast as that of those on diet 1. The rats were uniformly bad-tempered and excitable and several attempted to bite the writer while being dosed. Seekles & Sjollema [1933] have shown that low Ca:P ratio diets increase the excitability of the muscles. None of these rats had rickets and the line test gave no value lower than 5. The bone ash figures were, however, lower than normal, indicating a fair degree of osteoporosis, and varied between 38·73 and 49·21 %. The administration of vitamin D had no effect upon these values.

The negative control animals all showed in their teeth the same signs of malcalcification as were found with diet 2. The dentin response to vitamin D was qualitatively the same in all teeth. As with diet 2, the lingual side reacted by the laying down of a zone of deeply staining dentin, which became wider the longer the vitamin had acted and was often made up of incremental stripes. On the labial side, however, an entirely different picture was found. Here the dentin responded by the formation of large clumps of interglobular dentin over the whole predentin area. With higher dosages these tended to fuse and form a more homogeneous mass, especially on the odontoblast side, but even when this happened the predentin was always abnormally wide (Fig. 11).

Three litters of rats were treated with procedure (*a*). The response to NaF in the rats getting no vitamin D appeared as a fine blue line in the extremely wide labial predentin and measurements showed that calcification was delayed from the normal 24 hr. to from 15 to 20 days (Fig. 12). Calcification was not delayed to the same extent on the lingual side, but in one tooth a similar line was seen in the predentin on this side also. The incremental dentin growth rate in these teeth was 48μ during the last 10 days of the experiment. The administration of vitamin D increased the incremental growth of the dentin to 76μ over the last 10 days of the experiment, a value still much below normal. The irregular deposits of labial dentin made it quite impossible to compute the time of action of the vitamin by the methods used with the rats on diet 2. It could be seen, however, that the new interglobular dentin was deposited up to and even beyond the NaF line. Thus the clear predentin between the NaF line and the calcified dentin in Fig. 12 is filled in with interglobular dentin in Fig. 13, indicating the calcification of dentin formed before the NaF was injected. In this particular section predentin, laid down about 3 days before vitamin D dosage, had been retrospectively calcified. The reactive dentin on the lingual side was in some cases laid down in stripes. From evidence obtained using procedure (*c*) it will be seen that these stripes were daily increments of dentin. Using procedure (*a*) the vitamin had acted at this dose level for from 8 to 9 days.

Three litters of rats were treated with procedure (*b*). With the lower dose levels, no change was seen in the labial dentin, but with doses above 2·3 i.u. a response of the type already described was found, the whole predentin being filled with irregularly laid down interglobular dentin, which tended to fuse and fill up the predentin at the higher dose levels. On the lingual side, a purple zone of reactive dentin was seen at all dose levels, the number of stripes present in the teeth indicating that the vitamin had acted for from 5 to 9 days, depending on the dose level.

Five litters of rats were treated with procedure (*c*). Rats from two of these litters were killed at 24 hr. intervals after the vitamin had been given. On the labial side of the tooth no change was noted till 3 days had elapsed, when the whole predentin width began to fill up with interglobular dentin. In later sections the old predentin became completely replaced by interglobular dentin which tended in some cases to fuse into a solid line in the part nearest the pulp. Thus the labial predentin laid down from the time the vitamin had been given could retrospectively calcify 3 days later; and, since it has also been found that predentin laid down 3 days before the vitamin had been given could be similarly affected, this retrospective calcification could involve predentin laid down as much as 6 days previously. On the lingual side a narrow strip of new dentin was visible 2 days after vitamin dosage. This strip was present as two stripes 3 days after dosage, and in the teeth of animals killed on successive days one more stripe was added per day. This showed that these stripes were daily increments of dentin. These results also suggested that the vitamin had begun to act only a few hours after dosage, since the new dentin would not form till it had existed for 24 hr. as predentin.

In order to determine more accurately when vitamin D had begun to act, the rats of three litters were killed at 6-hourly intervals, up to 72 hr. after vitamin D dosage. No response was found in the labial dentin till 72 hr. had elapsed, but the lingual dentin showed a fine calcified line 30 hr. after dosage (Fig. 14). From this it may be concluded that the vitamin began to act on lingual predentin formation about 6 hr. after it had been given.

Discussion

The Steenbock & Black diet is undoubtedly deficient in many respects and growth on this diet and its modifications is known to be poor. Carnes, Pappenheimer & Stoerk [1942] conducted experiments in which the Steenbock & Black diet without $CaCO_3$ was the basal ration as in the present experiment. To this Ca and P salts were added to give various ratios and contents. In all cases they found growth to be poor even although a vitamin B complex concentrate was also given. Zucker, Hall & Young [1941] stated that the addition of P to the Steenbock & Black diet increased the growth rate over that of animals on the diet alone. The present results confirm this but even so the growth rate was very slow.

Normal calcification will, however, occur if the Ca:P ratio is adjusted. Karelitz & Shohl [1927] found a rapid healing of rickets in rats on the Steenbock & Black diet if phosphate was added. Key & Morgan [1932] likewise reported that when the $CaCO_3$ of this diet was replaced by calcium phosphate complete healing of rickets took place in 10 days. In the present experiments the animals eating diet 1 were maintained on this over the whole experimental period. As has been found by other workers [Sherman & Macleod, 1925; Yeager & Winters, 1935] in stunted animals, the chemical composition of the bones was superior to that of the weight controls, but inferior to that of the age controls. Examination of the teeth showed that almost normal calcification occurred with the diet alone, and completely normal calcification when vitamin D was added. Since this diet contained adequate Ca and P, it would appear, in spite of statements to the contrary, that rats do require a small amount of this vitamin, probably about 0·5 i.u. per day, for perfect calcification.

The changes in the teeth of rats on diet 2 after vitamin D dosage were of a quantitative nature and could be fairly accurately measured. They were similar to those reported by Schour & Ham [1934] and Schour *et al.* [1937], who gave normal and parathyroidectomized rats doses of parathormone and massive doses of calciferol (up to 644,000 i.u.). These writers reported the appearance of new deeply calcified dentin as a result of this procedure, the onset being almost immediately after the administration of vitamin D, as would be expected with such high dosage. They also stressed the appearance of two stages in the dentin response—a primary hypocalcified stripe and a secondary hypercalcified zone. Schour & Ham [1933] correlated these with changes in the serum Ca level, the former occurring during a rise in this figure, and the latter while the serum Ca was falling. No such zones were seen in the present work. The reactive dentin was preceded by a hypocalcified area but this was the uncalcified predentin already present before the vitamin was given. It is possible that the primary hypocalcified stripe only occurs with large doses of the vitamin. Schour *et al.* [1937] also commented on the prolonged improving effects of the vitamin which has also been found in the present results using very much smaller amounts of vitamin D. The histological picture after injection of parathyroid extract was the same as that after giving calciferol. It would appear probable that any agent affecting calcification would produce the same type of response in the dentin of animals on diets with a high Ca:P ratio. It is now well recognized that vitamin D does not act through the parathyroids.

Much attention has recently been paid to the formation of enamel, and the results obtained in the present work with diet 2 show that in many respects enamel and dentin calcification resemble each other. While the various theories of enamel formation differ in detail, they all agree that enamel matrix in man takes a definite length of time to mature [about 100 days, Chase, 1935; Diamond & Weinmann, 1940]. Diamond & Weinmann [1940] considered that calcification occurred only when the matrix was completely formed, possibly by a process of crystallization of contained colloidal Ca, this coinciding with the reduction in the enamel organ cells. These writers also made the important suggestion that hypoplastic changes in the enamel occurred during amelogenesis, i.e. during formation of the organic matrix and not during the subsequent calcification.

It is possible that such a theory applies equally to dentin formation with high ratio diets. Under normal circumstances predentin matures in 24 hr. and becomes calcified. With high Ca:P ratio diets maturation may be delayed as much as 8 days, but the calcification potentialities of the predentin formed under such circumstances cannot be altered by such agents as vitamin D or parathormone. Only the predentin formed after such agents are given is affected and reverts to the normal maturation cycle. At this time predentin is presumably being calcified in two separate places—one being at the edge of the old wide predentin formed before the agent was given, and the other in the reactive zone due to the agent. The rate of maturation of the old predentin is outside the influence of the calcifying agent.

The changes in the teeth of rats on diet 3 after vitamin D had been given differed on the labial and lingual sides. Different reactions on the two sides of the tooth have been previously described in other conditions, such as vitamin A deficiency [Wolbach & Howe, 1925, 1933; Irving & Richards, 1939; Schour, Hoffman & Smith, 1941] and Mg deficiency [Gagnon, Schour and Patras, 1942]. The reaction on the lingual side was the same as that of the rats on diet 2, a new reactive zone of dentin being formed. On the labial side, however, an entirely different picture was seen.

The histological appearance on the labial sides of the teeth of the control animals on diets 2 and 3 were identical, with wide predentin and the usual signs of malcalcification. The intimate reactions of malcalcification and recalcification must differ considerably in the two cases since retrospective calcification could occur for up to 6 days in dentin formed in rats on low ratio diets. It is not easy to explain this difference. Dentin formed on the two diets differs in chemical composition. Karshan & Rosebury [1932] and Gaunt & Irving [1940] found that the ash content of the dentin of rats on high Ca:P ratio diets was not altered, but that of rats on low Ca:P ratio diets was lowered in amount. Karshan & Rosebury [1933] further reported that the effects produced by low Ca diets were irrespective of the P content of the diet. It would thus appear that with a low intake of Ca and a low blood Ca value, such as these rats must have had since they were so liable to tetany, a different kind of dentin is formed. The process of maturation of this dentin can be accelerated by the vitamin in a way not possible on other diets. If this be conceded, then it must be admitted that Diamond & Weinmann's theory [1940] of hypoplasia does not apply to this type of dentin, since the potentialities of calcification are not irreversibly laid down in the matrix. It is not possible to see at present any explanation of this apparent dental heresy, but the facts cited in this paper cannot bear any other interpretation. Karshan & Rosebury [1933] found that the administration of cod liver oil to rats on low ratio diets improved the calcification of the incisors but the dentin lining the pulp cavity became very irregular, a finding similar to that reported in the present paper.

The changes in the teeth after vitamin D acted as an accurate and the earliest, so far described, index of the action of the vitamin. It would appear from the literature that about 3 days are the minimum for the bony response to occur in rachitic rats after vitamin D dosage. Thus Bills, Honeywell, Wirick & Nussmeier [1931] found that a certain amount of vitamin D (probably between 20 and 30 i.u.) gave their grade of 2+ healing after 14 days. The same amount of healing could be obtained after 3 days, but 105 times the amount of the vitamin had to be given to achieve this. They further stated that 5 days was the minimum curative period for a satisfactory line test assay. Kramer, Shear & Siegel [1931] found that healing could be detected in the epiphyses of some rats 3 days after the daily dosage with about 3 i.u., but chemical changes in the blood (a rise in Ca × P) had occurred earlier than this. Morgareide & Manly [1939] stated that the appearance of the line test coincided with the occurrence of radio-P in the epiphysis, when this had been given concurrently with the vitamin, and that this occurred 54–72 hr. after dosage with 10 or 15 i.u. In the present experiments no epiphyseal response was detected till at least 5 days after vitamin administration.

Evidence of vitamin action could be detected in the teeth in the present experiments very much earlier than in the bones by any of these cited workers and using doses of vitamin D much smaller than those of Bills *et al.* It would thus appear that the teeth are a far more sensitive index of vitamin D activity than the epiphyses. This confirms the findings of previous workers that the teeth are more avid of calcifying agents than the bones [Karshan & Rosebury, 1932; Gaunt, Irving & Thomson, 1939]. It is of interest that Kramer *et al.* detected changes in the blood Ca and P at least a day before histological changes in the bones were noted; while Bevelander & Hoskins [1939] considered the teeth changes to be even more delicate indices of alterations in Ca and P metabolism than the serum values.

Schour *et al.* [1937] stated that a reaction in the incisor of the parathyroidectomized rat could be seen 24 hr. after dosage with from 46,000 to 644,000 i.u. of calciferol. These results, obtained with such massive dosage, hardly compare with those reported here.

The present theories of vitamin D action do not agree as to where the vitamin exerts its action. Nicolaysen [1937 *a*, *b*] produced very convincing evidence that vitamin D acted upon Ca absorption in the gut, an extension of the theory of Harris & Innes [1931], P absorption being secondarily improved. Workers with radio-P have not, however, found that vitamin D had any effect upon P absorption [Dols, Jansen, Sizoo & de Vries, 1937; Morgareide & Manly, 1939] and in addition the latter workers objected that vitamin D could not act solely by improving intestinal absorption, as the time of its action would not allow of this. After the addition of P to a high Ca:P ratio diet, healing is detectable within 12 hr. [Kramer *et al.* 1931] or 24 hr. [Karelitz & Shohl, 1927], whereas after vitamin D dosage, 54 hr. was the minimum time for healing to be first seen. They therefore concluded that vitamin D required a certain interval of time in which 'to mobilize the phosphorus required for the healing process', and further that the 'mode of action of vitamin D is not limited to the control of intestinal absorption of the elements concerned in calcification'. Other workers have made similar suggestions. Thus McGowan, Cunningham & Auchinachie [1931] and McGowan [1933] considered that vitamin D liberated P from phospholipins in the body. Schneider & Steenbock [1939] stated that the vitamin could act by depriving the soft tissues of P which was transferred to the calcifying sites, though Day & McCollum [1939] have criticized this view.

The present results do not support the theory that vitamin D acts solely on intestinal absorption. It will be seen that while the vitamin could act within 24 hr. with fairly large doses, as much as 8 days could elapse before it began its action on the teeth when given in small doses. It is hard to conceive of the vitamin remaining for this length of time in the gut before starting its action, and it would appear most unlikely that it acts solely in this part of the body. From what other part of the body it mobilizes the elements necessary for calcification to begin it is at present impossible to say.

In the present results the speed of onset of new calcification was approximately the same with diets 2 and 3, using doses of 18·4 i.u. With diet 2 the response was progressively later when lower doses were given; with diet 3 the response was also probably later with smaller dosage, but accurate estimation was not possible. With diet 1 the vitamin acted in 24 hr. at all dose levels which gave a response. These findings would suggest that a certain state has to be attained in the body before vitamin action begins; this state is more rapidly developed with normal ratio diets, of greater dosage of vitamin D. This suggestion is reminiscent of the work of Kramer *et al.* [1931] who found an increase in the blood Ca × P before vitamin D had actually begun to act on the epiphyses, though it has been pointed out [Reed, Struck & Steck, 1939] that the blood changes in rickets are inconstant and not a reliable guide to the metabolic changes. From a consideration of the literature it would appear very probable, however, that this state which precedes the onset of the healing process is some kind of mobilization of Ca and P. As is well known, Robison & Soames [1924] and Shipley, Kramer & Howland [1926] found that rachitic bones would calcify *in vitro* if immersed in fluids of appropriate composition; while Karelitz & Shohl [1927], Kramer *et al.* [1931], Key & Morgan [1932] and Nicolaysen & Jansen [1939] have found that high Ca low P rickets could be healed by the administration of phosphate.

Whatever the fundamental nature of this mobilization, it would appear from the present work that it requires a definite interval of time to be attained, depending on the diet and dosage of vitamin D; once it has been reached it exerts a maximal effect whether it begins early or late.

SUMMARY

1. Young rats weighing from 50 to 60 g. were placed on diets of different Ca:P ratio, constructed by modifying the Steenbock and Black rachitogenic diet no. 2965, for 28 days. They were then dosed with vitamin D and some were also injected with NaF solution to act as a marker on the tooth. After a further period they were killed and examined. The response in the dentin of the incisor teeth was such that the time of action of the vitamin could be computed.

2. Rats on the diet with a Ca:P ratio of 1·8 were stunted in size but the bone ash was normal. Their incisor teeth were very slightly malcalcified. Administration of vitamin D caused completely normal tooth formation. Vitamin action was detectable after 24 hr. The results suggested that rats require about 0·5 i.u. per day for optimal calcification.

3. Rats on the diet with a Ca:P ratio of 4·4 showed all the signs of rickets and had malcalcified teeth. Administration of vitamin D caused a resumption of new calcification in the dentin, the dentin matrix laid down before dosage being unaffected. A logarithmic relationship was found between the response in the teeth and the dose level of the vitamin, as also occurs with the line test.

The teeth were much more sensitive to the action of the vitamin than the epiphyses. No epiphyseal response was found in any rat till the vitamin had acted for at least 4 days on the teeth. With moderately large doses of the vitamin (18·4 i.u.) a response was detected in the dentin after 24 hr.

4. Rats on the diet with a Ca:P ratio of 0·29 had a lowered bone ash but the epiphyses were normal in appearance. The teeth were very poorly calcified. The administration of vitamin D caused the same resumption of calcification on the lingual side of the tooth as was seen with high Ca:P ratio diets. The labial side of the tooth responded by an irregular deposition of interglobular dentin affecting dentin matrix laid down as much as 3 days before the vitamin was given. With doses of 18·4 i.u. vitamin D action was detectable after 30 hr.

5. These results are discussed with relation to current theories of tooth formation and of vitamin D action. It is considered that the vitamin action is not restricted to its effect upon intestinal absorption.

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Fig. 3.

Fig. 4.

Fig. 5.

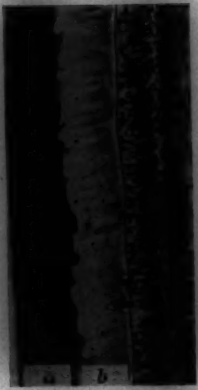

Fig. 6.

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EXPLANATION OF PLATES 1, 2 AND 3

a = dentin, b = predentin, c = odontoblasts, d = pulp.
Magnification in all microphotographs is × 102.

PLATE 1

Fig. 3. Longitudinal section of the intermediate part of the labial side of the upper incisor of a normal rat, to show the various structures.

Fig. 4. Longitudinal section of the intermediate part of the labial side of the upper incisor of a rat 38 days on diet 2, which was injected with 0·4 c.c. 2 % NaF solution and given 9·2 i.u. vitamin D 10 days before being killed. a^1 = dentin and predentin formed before the vitamin was given or had acted. a^2 = dentin formed under the influence of the vitamin. The arrow points to the NaF line. Note the incremental stratification of the new dentin.

Fig. 5. Longitudinal section of the intermediate part of the labial side of the upper incisor of a rat on diet 1, which was given 18·4 i.u. vitamin D 10 days before being killed. a^1 = dentin formed before the vitamin was given or had acted. a^2 = dentin formed under the influence of the vitamin. Note the better formation of dentin after the vitamin had been given.

Fig. 6. Longitudinal section of the intermediate part of the labial side of the upper incisor of a rat 38 days on diet 2. Note the very wide predentin and vascular inclusions.

PLATE 2

Fig. 7. Longitudinal section of the intermediate part of the labial side of the upper incisor of a rat 38 days on diet 2, which was given 9·2 i.u. vitamin D 10 days before being killed. a^1 = dentin and predentin formed before the vitamin was given or had acted. a^2 = dentin formed under the influence of the vitamin.

Fig. 8. Longitudinal sections of the extreme proximal ends of the labial sides of teeth of rats on diet 2. *a*, killed 1 day after dosage with 18·4 i.u. vitamin D. No change in the predentin is seen. *b*, killed 2 days after similar dosage. A fine mass of calcospherites is seen in the predentin. *c*, killed 3 days after similar dosage. A large mass of dentin is now formed.

Fig. 9. Longitudinal section of the intermediate part of the labial side of the upper incisor of a rat on diet 2, killed 5 days after dosage with 18·4 i.u. vitamin D. Note the line of calcospherites in the predentin.

Fig. 10. Longitudinal section of the intermediate part of the labial side of the upper incisor of a rat on diet 2, killed 6 days after dosage with 18·4 i.u. vitamin D. The calcospherites have fused to form a solid line of new dentin.

PLATE 3

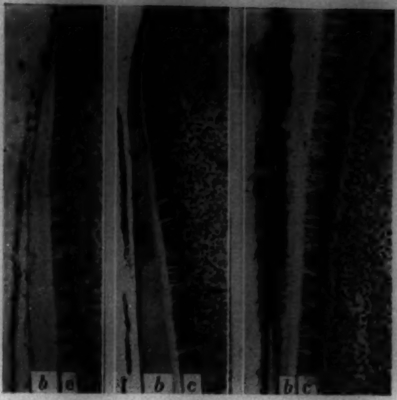
Fig. 11. Longitudinal section of the intermediate part of the labial side of the upper incisor of a rat 38 days on diet 3, killed 10 days after dosage with 18·4 i.u. vitamin D. Note the irregular and massive response in the dentin. It is impossible in this section to say at what stage the vitamin began to act.

Fig. 12. Longitudinal section of the intermediate part of the labial side of the upper incisor of a rat 38 days on diet 3. 0·1 c.c. 2 % NaF solution was injected 10 days prior to death. The arrow points to the fine line in the predentin caused by the NaF.

Fig. 13. Longitudinal section of the intermediate part of the labial side of the upper incisor of a rat on diet 3 for 38 days, killed 10 days after injection with 0·1 c.c. 2 % NaF solution and dosage with 9·2 i.u. vitamin D. The arrow points to the NaF line, which can be seen extending through the new dentin and as a fine black line in the predentin in the gap in the dentin at the top of the section. a^1 = dentin formed before the vitamin acted. a^2 = dentin formed under the action of the vitamin.

Fig. 14. Longitudinal section of the intermediate part of the lingual side of the upper incisor of a rat on diet 3, killed 30 hr. after dosage with 18·4 i.u. vitamin D. The arrow points to the fine line of well calcified new dentin.

Fig. 7.



a b c

Fig. 8.

Fig. 9.

Fig. 10.

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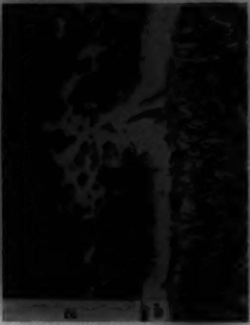

Fig. 11

Fig. 12.

Fig. 13

Fig. 14.

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THE NATURE OF SYNAPTIC TRANSMISSION IN A SYMPATHETIC GANGLION

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It has been shown that synaptic transmission in the stellate ganglion is largely, if not entirely, mediated by the local catelectrotonic potential which preganglionic impulses set up in ganglion cells—the synaptic potential [Eccles, 1943]. The synaptic potential is thus homologous with the end-plate potential of striated muscle. In the present paper the synaptic potential has been further investigated both in the normal and curarized ganglion, and with single and repetitive stimulation. In particular the effect of eserine has been studied in detail in order to throw light on the role of acetylcholine in setting up the synaptic potential and the discharge of impulses by the ganglion cells. The evidence so obtained on the acetylcholine action at the synapse is discussed in relation to other relevant data on synaptic transmission.

The method of experiment has been fully described in a preceding paper [Eccles, 1943]. All experiments were performed on cats under nembutal anaesthesia, which has no appreciable action on the sympathetic ganglion [Eccles, 1935*b*; Bronk, Tower, Solandt & Larrabee, 1938]. All injections of eserine were preceded by an injection of at least 1 mg./kg. of atropine.

A. THE ACTION OF ESERINE ON THE SYNAPTIC POTENTIAL

1. *Single and double preganglionic volleys in curarized ganglion*

In Fig. 1 synaptic transmission through the stellate ganglion has been blocked by curare; hence a single preganglionic volley sets up only a local negative potential of the ganglion cells relative to their postganglionic nerve fibres—the synaptic potential [Eccles, 1943]. Careful comparison of record (i) with (ii), (iii) and (iv) shows that the time course of the synaptic potential has not been significantly altered by even the largest dose of eserine. In some experiments, however, eserine has had a slight but significant action in lengthening the time course of the synaptic potential. Results from the experiment showing the largest effect are plotted in Fig. 2. When the respective potential scales are suitably adjusted, the initial rising phases are virtually identical, but the summit is delayed, and the falling phase slower. This effect appears more marked with a large dose of eserine (6·5 mg./kg.) than with 0·5 mg./kg. A much greater effect of the same general character was invariably produced by eserine on the end-plate potential of curarized muscle [Eccles, Katz & Kuffler, 1942, Fig. 7].

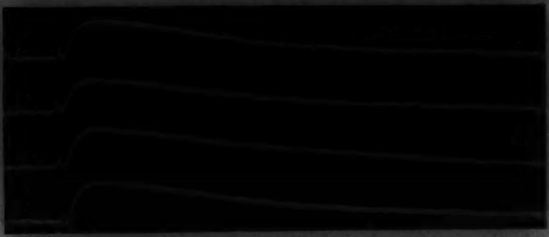

Fig. 1. Synaptic potentials set up in the curarized stellate ganglion by single preganglionic volleys: (i) before eserinization; between (i) and (ii) 0·5 mg. eserine/kg. injected intravenously; between (ii) and (iii), and (iii) and (iv) further doses of 2 mg./kg. At the same time small doses of curare were injected in order to maintain the complete synaptic block. The base-line for each record has been drawn in order to facilitate comparison.

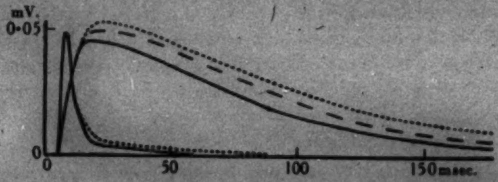

Fig. 2. The slow rounded curves show synaptic potentials set up in another curarized ganglion, the continuous line before eserine, the broken and dotted lines after injection of 0·5 and 6·5 mg./kg. eserine respectively. Potential scale for dotted curve 0·8 time that shown. The continuous and dotted peaked curves have been determined by analysis of the corresponding synaptic potentials (see Discussion).

When the synaptic potential exceeds a critical value, an impulse is discharged from some ganglion cells, as shown by a spike superimposed on the synaptic potential [Eccles, 1943, § 4]. No significant alteration of this threshold potential has been observed with even the largest doses of eserine.

A second preganglionic volley, at intervals up to about 0·2 sec. after the first, adds a synaptic potential which is usually slightly larger than the first, but which may be much larger or slightly smaller [Eccles, 1943, § 5*a*]. Whatever the effect in any particular experiment, it is not significantly altered by even the largest doses of eserine.

2. *Repetitive preganglionic volleys in curarized ganglion*

Fig. 3 shows the synaptic potentials produced by repetitive preganglionic volleys at various frequencies in the deeply eserinized stellate ganglion

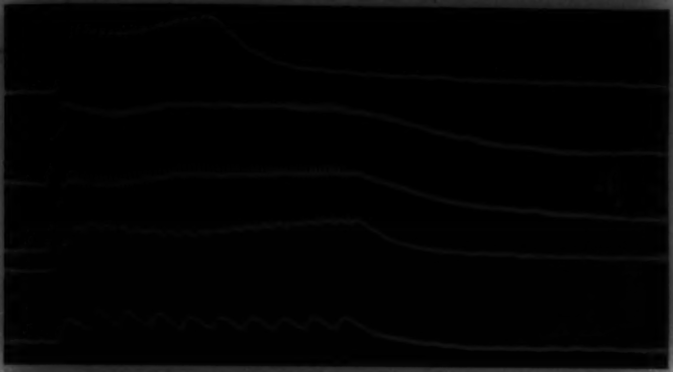

Fig. 3. Synaptic potentials of curarized and eserinized (6 mg./kg.) stellate ganglion set up by repetitive preganglionic stimulation; observations (i)–(v) respectively—55 per sec. for 0·35 sec.; then 140, 84, 28 and 13·5 per sec. for 0·7 sec. Time, 10 msec.

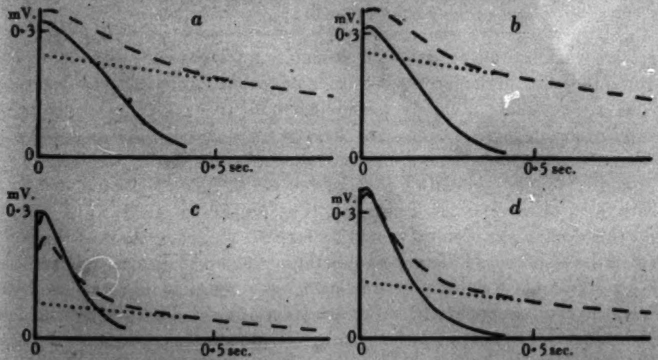

Fig. 4. Plottings of potentials of Fig. 5*a* [Eccles, 1943] as continuous lines, and of Fig. 3 as broken lines to show effect of eserine on time course of decay. Dotted lines described in text. Zero time corresponds to last stimulus of the repetitive series. *a*, 140 per sec., 0·7 sec.; *b*, 84 per sec., 0·7 sec.; *c*, 28 per sec., 0·7 sec.; *d*, 55 per sec., 0·35 sec. In order to facilitate comparison of non-eserinized and eserinized curves, the potential scale of latter is 0·59 time that shown.

(6 mg./kg.). The synaptic potentials set up in this same stellate ganglion by a similar series of volleys previous to eserinization have already been illustrated [Fig. 5a, Eccles, 1943]. From these two series Fig. 4 has been constructed in order to facilitate comparison of the decaying phases for corresponding responses.

The response at 13·5 per sec. resembles the single response in not being significantly modified by eserine either during the stimulation or the subsequent decay. On the other hand, with 28 per sec. and still more with 84 and 140 per sec. (all 0·7 sec. duration) the eserinized ganglion gives a prolonged negative response on cessation of stimulation. However, with 28 and 55 per sec. there is still the incomplete fusion of the successive synaptic potentials giving a wavy plateau closely resembling the corresponding response before eserinization.

Fig. 5. As in Fig. 3 in another experiment after 6 mg. eserine/kg.; all series 0·4 sec. stimulation at respectively 125, 80, 37 and 25 per sec. Time, 10 msec.

Before eserinization the synaptic potentials showed, during the last 0·4 sec. of rapid repetitive stimulation (84 and 140 per sec.), a progressive decline to about 80% of the maximum potential [Fig. 5a, Eccles, 1943]. Fig. 3 shows that this decline has been prevented by eserine. This suggests that this latter part of the response is lifted by a gradual building up of that negativity which becomes evident at the end of stimulation on account of its very slow decay. Thus, there would appear to be a separation of the synaptic potential built up during repetitive stimulation into quickly decaying and prolonged fractions. This separation is more clear-cut in Fig. 5, where the prolonged negative potential runs a still slower time course than in Fig. 3. In fact the rate of fall in the record is no faster than could be accounted for by the time constant of the amplifier—half-time equals 6 sec. for a constant potential applied in series with the preparation. In Fig. 6a the complete course of the response to 125 per sec. from Fig. 5 has been plotted, together with the similar responses before eserinization and after injection of 1 mg. eserine/kg. body weight. Again, the course of the response during tetanization suggests that the prolonged fraction is being built up during tetanization. The very slow decay of this prolonged potential makes it possible to extrapolate it back to the end of the stimulation as shown by the dotted line in Fig. 6*a*. Similar extrapolations are tentatively shown in Fig. 4.

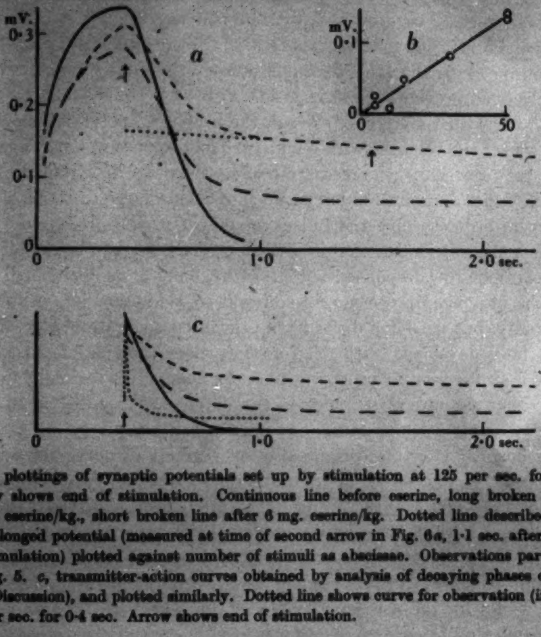

Fig. 6. *a*, plottings of synaptic potentials set up by stimulation at 125 per sec. for 0·4 sec. Arrow shows end of stimulation. Continuous line before eserine, long broken line after 1 mg. eserine/kg., short broken line after 6 mg. eserine/kg. Dotted line described in text. *b*, prolonged potential (measured at time of second arrow in Fig. 6*a*, 1·1 sec. after cessation of stimulation) plotted against number of stimuli as abscissae. Observations partly shown in Fig. 5. *c*, transmitter-action curves obtained by analysis of decaying phases of Fig. 6*a* (see Discussion), and plotted similarly. Dotted line shows curve for observation (iii), Fig. 5, 37 per sec. for 0·4 sec. Arrow shows end of stimulation.

When the negative synaptic potential is thus divided into two fractions, it is seen that the more quickly decaying fraction closely resembles in time course the non-eserinized after-potential. It has been shown that, after more than 15 stimuli at a frequency above 50 per sec., the synaptic potential of the non-eserinized ganglion exhibits an initial period of slow decay [Eccles, 1943, § 5*b*], which later quickens until, after about 0·3 sec., it is almost as rapid as with responses to single stimuli or low frequencies of stimulation. This initial slow decay is evident in the non-eserinized synaptic potentials in Figs. 4 and 6*a*, and the quickly decaying fraction of the corresponding eserinized potential exhibits an almost identical period of initial slow decay. This similarity has been observed with all repetitive responses, the degree of initial slowing in the non-eserinized responses to different frequencies and durations being closely mimicked in the eserinized responses, no matter how deep the eserinization (up to 10 mg. eserine/kg. body weight).

The striking action of eserine on the repetitive synaptic potentials is thus entirely due to the appearance of a prolonged type of synaptic potential, which has not been observed with the longest high-frequency responses of the non-eserinized ganglion—usually 140 per sec. for 0·7 sec., but in one experiment 130 per sec. for 5 sec. was tried. On this prolonged after-potential of the eserinized ganglion there is superimposed the same brief after-potential that is observed previous to eserinization.

In Fig. 6*a*, with 1 mg./kg. of eserine, the prolonged potential is only about half the size of the response with 6 mg./kg. The response at 3 mg./kg. is about midway between. Thus, the maximum effect may not be attained even with 6 mg./kg. Other experiments have also shown that large doses of eserine are necessary in order to give the full development of the prolonged potential, e.g. with 2 mg. eserine/kg. the prolonged responses were about half the size of the corresponding responses in Fig. 3 with 6 mg./kg. With small doses of eserine the prolonged responses, in addition to being smaller, usually decay more rapidly, but base-line drifts have prevented accurate observations.

When fully developed with deep eserinization, the size of the prolonged potential is approximately proportional to the number of preganglionic volleys, provided that the rate of stimulation is not too slow, e.g. below 20 per sec., for effective summation to occur, or so rapid, e.g. 140 per sec., that impulses cease to be fully effective (presumably on account of nerve refractoriness). For example, in Fig. 6*b* an approximately straight-line relationship obtains for the observations of Fig. 5, and it has often been observed that halving the duration of stimulation approximately halves the size of the prolonged potential. No accurate determinations have, however, been possible because, on account of respiratory and cardiac movements, the base-line has not been steady enough for the necessary length of time—1–2 sec. The slow drifts of the base-line may be as large as 0·04 mV./sec., but usually it is steady to about 0·01 mV./sec. Even that would represent a serious error in Fig. 6*b*, where the potentials have been measured relative to the base-line obtaining at the commencement of stimulation 1·5 sec. earlier.

Comparison of the non-eserinized with the eserinized synaptic potentials in Figs. 4 and 6 indicates that the prolonged fraction tends to submerge the quickly decaying fraction. For example, with Fig. 4 *c*, *d* the quickly decaying fraction is much larger relative to the non-eserinized potential than it is in Fig. 4 *a*, *b*, and, when the tetanization at 140 per sec. was continued for 4 sec. or longer, the quickly decaying fraction was completely submerged. Partial submergence is also evident in Fig. 6. In another experiment on the deeply eserinized ganglion, the quickly decaying fraction was almost completely submerged after only 0·8 sec. tetanization at 140 per sec. Further information on this submergence is provided by a comparison of the courses of the non-eserinized and the eserinized potentials during stimulation, from which the production of the slowly decaying fraction has already been inferred. The addition observed during the course of stimulation is found to be much less than the prolonged potential appearing subsequent to the stimulation (cf. Fig. 6*a*). This suggests that the prolonged and quickly decaying fractions of the synaptic potential mutually occlude each other, and hence arise in part at a common locus. This spatial overlap is, of course, to be expected, for, as seen above, synaptic potentials spread electrotonically even along the postganglionic fibres [Eccles, 1943].

B. RESPONSES OF NORMAL GANGLION TO PREGANGLIONIC STIMULATION

1. *The spike potential*

The simple (S_2) type of spike response in the cardiac branches of the stellate ganglion makes it particularly suitable for the study of impulse transmission through a sympathetic ganglion [cf. Bronk, Tower & Solandt, 1935; Bronk *et al.* 1938; Bronk, 1939]. The present experiments have confirmed the close association of inhibition with after-positivity of the ganglion cells [cf. Eccles, 1935*c*, 1936*a*]. As Lloyd [1939] has pointed out, the contrary findings of Rosenblueth & Simeone [1938*a*] are due to their failure to distinguish between the spike and the after-negativity on which it is superimposed [cf. Rosenblueth, 1940]. When testing for impulse transmission it is essential to record from the postganglionic nerve [cf. Eccles, 1935*b*; Bronk *et al.* 1938; Bronk, 1939]. In regard to the transmission of repetitive trains of impulses through the stellate ganglion, the present experiments have confirmed the finding of Bronk & Pumphrey [1935] and Rosenblueth & Simeone [1938*a*] that, with frequencies above a critical rate,—usually about 50 per sec., there is a decline in the repetitive spike potentials (compare Fig. 12, showing very little decline at 47 per sec. with Fig. 7 (iii), where there is a rapid decline at 81 per sec.).

2. *The after-potentials*

It is generally agreed that the after-potential of the activated ganglion is compounded of two independent but overlapping potentials—usually called the negative and positive after-potentials [Eccles, 1935*c*, 1936*a*, 1937*a*; Obrador & Odoriz, 1936; Whitteridge, 1937; Rosenblueth & Simeone, 1938*a*; Bronk, 1939; Lloyd, 1937, 1939]. These potentials were originally called the *N* and *P* waves by the present author, because their identity with the negative and positive after-potentials of nerve had not been established. This terminology has been retained in this paper, for, despite the experimental support for such an identity [see Lloyd, 1939, for a summary], a large part of the *N* wave is shown by the following evidence to differ from the negative after-potential which arises secondarily to a nerve impulse, and to be a true synaptic potential, homologous with the end-plate potential of the neuro-muscular junction.

(*a*) During the refractory period following an antidromic volley, a preganglionic volley fails to set up the discharge of impulses from the ganglion cells; nevertheless it adds a large *N* wave to the antidromic response [Eccles, 1936*a*].

(*b*) Similarly, a preganglionic volley fails to set up the discharge of impulses from curarized ganglion cells, but still evokes a large negative potential, the synaptic potential, which closely resembles the *N* wave in time course and in associated facilitation [Eccles, 1943].

(*c*) Veratrine greatly increases and lengthens the negative after-potential of nerve, and a similar effect is observed with the ganglionic after-negativity [Lloyd, 1939; Rosenblueth, 1942]; both facilitation and the synaptic potential of the curarized ganglion are, however, altered little, if at all (unpublished observations). Similarly, though veratrine greatly increases the negative after-potential of muscle, it has no action on the end-plate potential either in normal or curarized muscle [Eccles *et al.* 1942; Kuffler, 1943*b*].

Comparison of the ganglionic potentials set up by a preganglionic and by an antidromic volley has shown that the ganglionic potential set up by a single preganglionic volley is partly due to impulses in the ganglion cells (the spike and the negative and positive after-potentials), and is partly a local negative potential directly produced by the preganglionic impulses (the synaptic potential). With the superior cervical ganglion a single preganglionic volley was thus shown to set up a large synaptic potential [Eccles, 1936*a*], particularly in the S_1 ganglion cells. This synaptic potential disappeared in about 200 msec., for after this time the after-potentials set up by preganglionic and antidromic stimulation were practically identical both in size and time course. Comparison of the responses set up in the stellate ganglion by single preganglionic and antidromic volleys (Fig. 8*a*) shows that the synaptic potential is much smaller, a difference presumably capable of correlation with the absence of S_1 ganglion cells.

Fig. 7 shows the ganglionic potentials set up by repetitive preganglionic stimulation for 0·72 sec. and also by a single stimulus. With 12·5 per sec. the after-positivity is more prolonged than with the single response, but with more rapid stimulation (81 and 132 per sec.) a negative wave appears to be superimposed on the initial part (about 0·3 sec.) of the after-positivity, which is itself still further prolonged. This superimposed negative wave often becomes so large that there is a brief initial phase of relative negativity (cf. Figs. 9 *a*, *c*, 10).

With repetitive ganglionic after-potentials, determination of the synaptic potentials by comparison with the corresponding antidromic potentials (cf. above for single responses) is not possible at high frequencies, because of the development of a progressive block in synaptic transmission—the so-called Wedensky inhibition. Thus the spikes of Fig. 7 show that, even at 81 per sec. there is a progressive decline in the number of ganglion cells activated preganglionically, though with antidromic stimulation the spike heights are well maintained even at 100 per sec. The synaptic block would, however, be much less than is indicated by the spike heights, for with rapid stimulation the ganglion cell discharge becomes progressively more asynchronous. For example, in Fig. 7 (ii) the large *P* wave of the after-potential shows that a large but unknown fraction of the ganglion cells was discharging throughout the stimulation, and yet the spikes had virtually disappeared after the first few stimuli. With rapid stimulation it is thus impossible by comparison with the antidromic responses to discover the time course of the after-potentials resulting from the discharge of impulses from the ganglion; hence the synaptic potential can only be approximately determined (cf. also Figs. 9, 10). The after-discharge occurring after rapid preganglionic stimulation (§ B3) further interferes with the comparison.

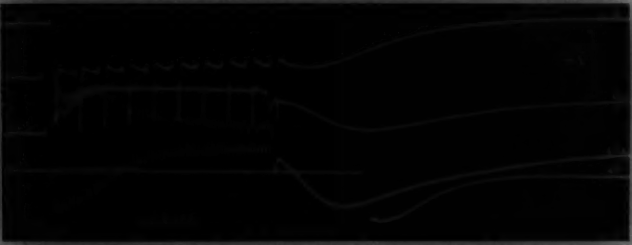

Fig. 7. Potentials set up in normal stellate ganglion (measured relative to postganglionic cardiac nerve) by preganglionic stimulation: (i), (ii) and (iii) respectively 12½, 132 and 81 per sec. for 0·72 sec.; (iv) single stimulus.

In Fig. 8 there are plotted, superimposed, the ganglionic after-potentials set up by similar preganglionic and antidromic tetanization at a slower frequency (50 per sec.), where the Wedensky block developed much more slowly. With the antidromic records the grid lead surrounds the postganglionic pole of the ganglion, the earthed lead is around the intact, but isolated, postganglionic nerve some 5 mm. distally, and the stimulating electrodes are applied several millimetres further distally. Responses to preganglionic stimulation were recorded without altering the position of the recording leads. Thus Fig. 8 shows potential, falling to 1/ε in about 1·7 mm. [Eccles, 1943]). When followed further postganglionically, however, no further decrement eventually occurs in the after-potential (recorded with one lead on the killed end), and it is then entirely a postganglionic after-potential [cf. Bronk *et al.* 1938; Bronk, 1939].

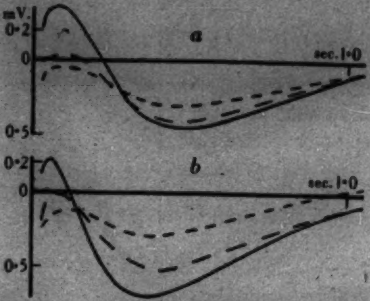

Fig. 10. *a*, after-potentials of normal stellate ganglion set up by preganglionic stimulation of 84 per sec. for 0·7 sec. Continuous line recorded from ganglion, long and short broken lines at 2 and 4 mm. respectively along the postganglionic nerve. Zero time corresponds to last stimulus. *b*, as in *a*, but after a stimulus of 84 per sec. for 0·19 sec.

3. *After-discharge*

Evidence for a small, but inconstant, after-discharge from the superior cervical ganglion was derived from a comparison of the nictitating membrane responses produced by identical preganglionic and postganglionic tetanization at a rate of 40 per sec. for 1–2 sec. [Eccles, 1935*a*], and such after-discharges have been directly recorded by Larrabee & Bronk [1938] and Bronk [1939]. In the present experiments it has been found that after-discharges are invariably set up after short bursts of rapid preganglionic tetanization, for example, by 25 stimuli at 130 per sec. (Fig. 11 (i)). The maximum intensity of after-discharge is usually attained after 50 stimuli (Fig. 11 (ii)), but often it is more prolonged with longer tetani, for example, the low intensity discharge of Fig. 11 (iv) persisted for several seconds. After-discharge is also usually seen with short bursts of stimulation at 80–90 per sec. (cf. Fig. 11 (v)), but stimulation at rates under 50 per sec. produces little or no after-discharge even when continued for long periods [cf. Bronk, 1939]. Thus in Fig. 11 (vi), (vii) and (viii) a small, brief after-discharge follows stimulation at 46 per sec. for 4·2 sec., but none with 25 per sec. for 4 sec. or 12 per sec. for 7 sec.

There is thus a correlation between the conditions for producing after-discharges and those building up large synaptic potentials. For example, in Fig. 9*a* there were after-discharges during the responses to 16, 31, 60 and 100 stimuli, and the time of cessation (marked on the curves) appears to be related to the decay of the synaptic potential. However, after-discharge is often observed when the synaptic potential fails to sum sufficiently to give a brief initial negative phase, e.g. in Fig. 7 (ii) there was a little after-discharge. Larrabee & Bronk [1938] suggested that the after-positivity was responsible for the brief early intermission in the after-discharge set up by prolonged stimulation. Intermissions brought about in this way are seen in Fig. 11, observations (ii) and (iii).

Fig. 11. After-discharges from normal stellate ganglion resulting from repetitive preganglionic stimulation. Arrow at lower left corner shows time of last stimulus for all records. The large diphasic potentials at about 0·5 sec. intervals are the electrocardiogram. (i)–(iv) stimulation at 130 per sec. for 0·2, 0·4, 0·8 and 4 sec. respectively; (v) 80 per sec., 0·8 sec.; (vi) 46 per sec., 4·2 sec.; (vii) 25 per sec., 4 sec.; (viii) 12 per sec., 7 sec. The noise level of the base-line is shown in these last two observations as no after-discharge occurred.

C. THE ACTION OF ESERINE ON THE NORMAL GANGLIONIC POTENTIALS

1. *The spike potential*

Rosenblueth & Simeone [1938*b*] state that eserine strikingly modifies the response of the ganglion to repetitive preganglionic stimulation, causing a progressive decrease in spike height at low frequencies (10 and 19 per sec. in their Fig. 7), where it does not normally occur. In the present experiments eserine in doses up to 8 mg./kg. has had no significant effect on spike heights during tetanic stimulation (cf. Fig. 12). Durations of stimulation up to 0·8 sec., and frequencies from 12 up to 130 per sec. have been employed in hundreds of records with uniformly negative results. Bronk *et al.* [1935] similarly failed to detect any action of eserine. The present negative findings may also be linked with the failure of Brown & Feldberg [1936] to find an 'inhibiting dip' on repetitive activation of a ganglion naturally circulated the differences between the after-potentials set up by preganglionic and antidromic stimulation.

With 9 stimuli at 50 per sec. (Fig. 8*b*) the latter parts of the *P* waves are closely similar (cf. the single responses of Fig. 8*a*), but with the preganglionic response (broken line) there is a large initial phase of added negativity. The synaptic potential has by summation been increased to several times the single response of Fig. 8*a*. This summation of synaptic potential is seen to be still further developed with 20 and 40 preganglionic stimuli (Figs. 8 *c*, *d*). However, on account of developing synaptic block (spike heights reduced to about 50% after 40 stimuli), comparison becomes increasingly unreliable with lengthening stimulation, as is reflected by the poorer matching of the latter parts of the *P* waves.

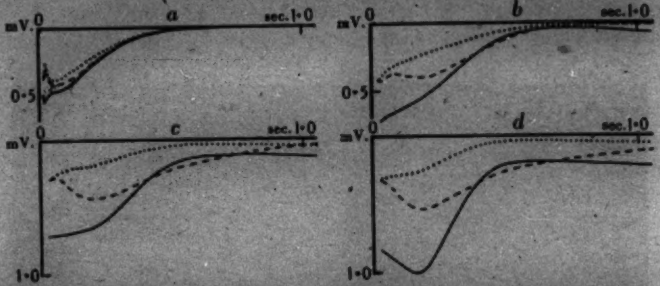

Fig. 8. After-potentials of stellate ganglion recorded with one electrode on the ganglion and one on the intact, but isolated, postganglionic trunk 5 mm. distally. The continuous and broken lines show respectively the after-potentials set up by similar antidromic and preganglionic stimulation. After eserinization (2·5 mg./kg.), preganglionic responses are shown by the dotted lines, but antidromic responses are not significantly altered. Zero time corresponds to last stimulus. *a*, *b*, *c* and *d* show respectively responses to 1, 9, 20 and 40 stimuli at a frequency of 50 per sec. All antidromic responses drawn on scale 0·46 times the scale shown for preganglionic responses, in order that latter parts of *P* waves are coincident for the non-eserinized single responses in *a*.

The building up of synaptic potential during repetitive stimulation varies considerably in different experiments (cf. Fig. 9 *a*, *b*). It is also greatly affected by the frequency of stimulation. Thus in Fig. 7, with stimulation at 12·5 per sec., there is but little summation of synaptic potential. Frequencies of 20–50 per sec. are transitional. The effect of stimulation frequency on summation of synaptic potential is typically shown in Fig. 9*c*, where the building up is less with 19 stimuli at 25 per sec. than with 17 at 38 per sec., and much less than with 17 stimuli at 88 per sec.; on the other hand, the *P* wave is not significantly changed.

The after-potentials set up by repetitive antidromic volleys always show the subdivision into an initial P_1 and a later P_2 wave separated usually by a distinct hump (cf. Fig. 8 *c*, *d*). With continued stimulation the P_1 wave increases and lengthens, eventually showing an initial phase of increasing positivity (Fig. 8*d*), while the P_2 wave increases approximately proportionally with the number of stimuli. However, it is important to realize that, on account of the method of recording, these antidromic potentials are not the ganglionic after-potentials but only the difference between the ganglionic and the postganglionic after-potentials.

Lloyd [1939] has summarized the evidence that the after-potentials recorded from a ganglion are largely produced in the ganglion cells and not in their postganglionic fibres [cf. Eccles, 1937 *a*]. The large after-potentials shown in Fig. 8 further support this conclusion. Since there is no reason to suppose that the potentials of the postganglionic fibres are significantly different during their intraganglionic course, the after-potentials recorded as in Fig. 8 must be very largely produced in the ganglion cells, for with the two leads the after-potentials of the postganglionic fibres would cancel out. In agreement with this the records of Fig. 10 *a*, 2 and 4 mm. from the ganglion, show that the *N* wave decrements rapidly along the postganglionic fibres and the *P* wave somewhat less rapidly. These spatial decrements are more rapid after shorter bursts of repetitive stimulation, e.g. with 0·19 sec. in Fig. 10*b*. Both in this respect and in the rate of spatial decrement, the postganglionic spread of the *N* and *P* waves in Fig. 10 is satisfactorily explained as an electrotonic spread of potentials produced in the ganglion (cf. the spread of the synaptic

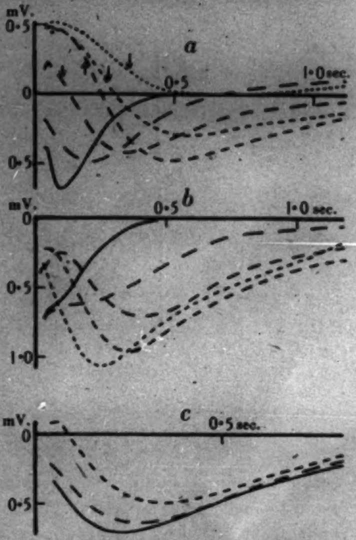

Fig. 9. *a*, ganglionic after-potentials for repetitive preganglionic stimulation at 132 per sec. Continuous line response to single stimulus; responses shown by progressively shorter broken lines for 8, 16, 31, 60 and 100 stimuli respectively. The arrows show the times of cessation of the after-discharges. Zero time corresponds to last stimulus. *b*, as in Fig. 9*a* in another experiment, but with response to 100 stimuli not shown. *c*, after-potentials plotted as in Fig. 9*a*, but with stimulation frequency varied: continuous line, 19 stimuli at 25 per sec.; long broken line, 17 stimuli at 38 per sec.; short broken line, 17 stimuli at 88 per sec.

potential, falling to 1/ε in about 1·7 mm. [Eccles, 1943]). When followed further postganglionically, however, no further decrement eventually occurs in the after-potential (recorded with one lead on the killed end), and it is then entirely a postganglionic after-potential [cf. Bronk *et al.* 1938; Bronk, 1939].

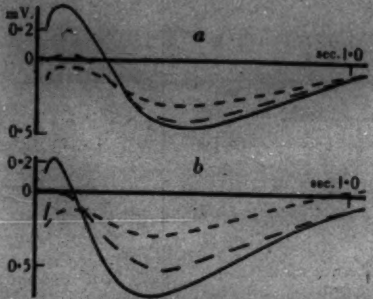

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Fig. 12. Ganglionic potentials set up as in Fig. 7 by preganglionic stimulation at 47 per sec. for 0·4 sec. (i) before eserinization, (ii) after injection of 8 mg. eserine/kg.

2. *The after-potential*

The effect of eserine on after-potentials has hitherto only been investigated with the responses to single or double preganglionic volleys. In some experiments of the present series no significant effect was observed [cf. Eccles, 1935*c*], but in others (cf. Fig. 8*a*) eserine produced a small decrease in the after-positivity [cf. Rosenblueth & Simeone, 1938*b*], which may have been due to a small increase and lengthening of the synaptic potential (cf. the effect of eserine on the synaptic potential, Fig. 2).

The intravenous injection, however, of even such a small dose as 0·2 mg. eserine/kg. produces a characteristic change in the ganglionic after-potentials set up by repetitive preganglionic stimulation. The N wave is increased and prolonged, and the P wave usually prolonged, as is typically shown in Fig. 13 *a*, *b* and *c*. A further dose of eserine accentuates these effects—particularly increasing the duration of the N wave, but, as Fig. 13 *a*, *b* and *c* shows, a maximum effect, at least for the N wave set up by brief tetani, is reached with a dose as low as 0·6 mg./kg., further increase to above 2 mg./kg. having little or no additional action.

The ganglionic action-potentials produced by antidromic stimulation throw light on the nature of this eserine action. Eserine even in doses up to 6 mg./kg. has no significant action on the antidromic after-potentials recorded from the ganglion; hence Fig. 8 shows clearly that the change in the after-potential is due to eserine greatly lengthening the synaptic potential set up by the preganglionic tetanus—up to at least 1 sec.—and this lengthened potential is larger with the longer tetani. The increased duration of the synaptic potential presumably provides the explanation of the much greater summation observed for the synaptic potentials set up by successive stimuli at slow rates of stimulation. For example, in Fig. 13*b*, summation of synaptic potentials with 12·5 per sec. for 0·8 sec. has become almost as great as with Fig. 13*a*, 130 per sec. for 0·1 sec., though before eserinization it showed little or no summation.

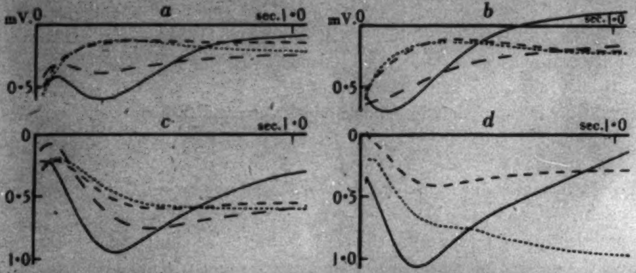

Fig. 13. Ganglionic after-potentials plotted as in Fig. 9 for repetitive preganglionic stimulation before (continuous line) and after graded doses of eserine (long broken line, 0·2 mg./kg.; shorter broken line, 0·6 mg./kg.; shortest broken line, 3·0 mg./kg.). Stimulation in *a* at 130 per sec. for 0·1 sec. (13 stimuli); in *b*, 12·5 per sec. for 0·8 sec. (10 stimuli); in *c*, 81 per sec. for 0·38 sec. (31 stimuli); in *d*, 81 per sec. for 0·75 sec. (60 stimuli); no record taken after 0·2 mg. eserine/kg.

It will be seen below that eserine greatly increases and prolongs the after-discharge from the ganglion cells. Summation of the *P* waves set up by this continued after-discharge would explain the great increase and prolongation of the *P* wave seen in Fig. 13*d* with full eserinization, and also in Fig. 13*c*. But after-discharge has never been observed after the few stimuli employed in Fig. 13 *a*, *b*, where the *P* wave also appears lengthened. Further comparative investigation with preganglionic and antidromic stimulation is indicated. Some experiments differ from those illustrated in Figs. 8 and 13—preganglionic stimulation setting up a negative ganglionic potential which persists for seconds. Presumably, the eserinized synaptic potential is so large and prolonged that it submerges the *P* wave.

In conclusion, all our findings show that eserine acts by increasing the duration of the synaptic potential, and hence the summation of the synaptic potentials set up by successive preganglionic volleys. This effect of eserine cannot always be detected after single or double preganglionic volleys, but rapidly increases with repetitive stimulation. The action of eserine in prolonging the *P* wave is due partly, but probably not entirely, to the after-positivities set up by the successive impulses of the after-discharge.

3. *After-discharge*

By using the nictitating-membrane response as an indicator, it has been shown that preganglionic tetanization (40 per sec. for 1–2 sec.) results in a prolonged after-discharge (15–60 sec.) from the eserinized superior cervical ganglion [Eccles, 1935*a*]. Rosenblueth & Simeone [1938*b*] recorded occasional brief after-discharges—the longest 100 msec. On the other hand, Bronk *et al.* [1935] only observed the depressing effect of high concentrations of eserine on synaptic transmission, presumably because the frequency of stimulation was too low.

Fig. 14*a* shows the invariable effect of progressive eserine dosage on the after-discharge produced by a brief preganglionic tetanus (132 per sec. for 0·75 sec.). By the use of very small coupling condensers the after-potentials are practically eliminated from the records, but on account of the high amplification the record only becomes visible at the end of the tetanization. The very small and brief after-discharge normally present (a few discharges ceasing in about 40 msec. in (i)) is increased and prolonged by 0·1 mg. eserine/kg. (ii), and is enormously affected by a further 0·2 mg./kg. (iii). Finally, 1 mg./kg. gives an after-discharge no more intense at the start, but continued with no sign of diminution during the 1·1 sec. of the record (iv). After 5 or more mg. eserine/kg. after-discharges have been observed to continue for as long as 10 sec. after the end of repetitive stimulation for 0·8 sec.

Not only does eserine increase and prolong the after-discharge set up by rapid repetitive stimulation, but it also causes it to occur with rates too slow to give after-discharges from the non-eserinized ganglion. For example, Fig. 14*b* shows after-discharge with 84 and 44 per sec. stimulation for 0·75 sec. in the eserinized ganglion (iii and iv), but none normally (i and ii); however, 27 per sec. for 0·75 sec. still fails to evoke an after-discharge (v). Even slower rates of stimulation give after-discharge if continued long enough. For example, in Fig. 14*c* (ii) stimulation at 12 per sec. for 4 sec. (48 stimuli in all) gives an after-discharge closely resembling that set up by 50 per sec. for 0·75 sec. (iii, 38 stimuli in all), while 25 per sec. for 3·8 sec. (iv, 95 stimuli in all) gave a much more intense after-discharge.

Thus, in the fully eserinized ganglion, the number of preganglionic volleys is the important factor determining after-discharge—not the rate of stimulation, which is the criterion for the non-eserinized ganglion (§ B3). The change-over from a frequency criterion to a number criterion has been repeatedly observed during progressive eserinization. The critical number of impulses for setting up an after-discharge in the fully eserinized ganglion (at least 5 mg./kg.) is about 20 (never less than 14) and is practically independent of frequency—frequencies lower than 12 per sec. have not been tried.

Fig. 14. After-discharges from stellate ganglion showing effect of various doses of eserine. In *a*, *b* and *d* arrow in lower left corner shows time of last stimulus. *a*, stimulation 132 per sec. for 0·75 sec. (100 stimuli): (i) before eserine; (ii)–(iv) after 0·1, 0·3, 1·3 mg. eserine/kg. respectively. Separate potential scale for (iv). Noise level shown by all but initial part of (i). *b*, (i) and (ii) stimulation at 84 and 44 per sec. for 0·75 sec. before eserine, and after 1·3 mg./kg. in (iii) and (iv) respectively; (v) 27 per sec. for 0·75 sec., 1·3 mg. eserine/kg. Separate potential scale for (iii)–(v). Noise level shown by (i), (ii) and (v). *c*, (i) base-line; (ii)–(iv) after-discharges set up by 12 per sec. for 4 sec., 50 per sec. for 0·75 sec. and 25 per sec. for 3·8 sec. respectively. 8 mg. eserine/kg. in all. Time, 10 msec. *d*, (i) base-line; (ii)–(iv) after-discharges set up by 132 per sec. for 0·75 sec.; eserine, 3 mg./kg. in all; (ii) before, and (iii) and (iv) after successive injections of curare, the second dose being twice the first.

The after-discharge is equally well observed with post-ganglionic recording, as with ganglionic recording, showing that the impulses are propagated normally along the postganglionic fibres. Recording from the stimulated preganglionic trunk (the thoracic sympathetic, caudal to the third white ramus) showed the presence of an after-discharge closely comparable in all respects with that recorded postganglionically. The following tests, however, on fully eserinized ganglia, show that this after-discharge is occurring in postganglionic fibres, passing from the stellate ganglion to the upper thoracic spinal nerves and not back along the stimulated preganglionic nerve fibres.

(1) Tetanic stimulation of the thoracic sympathetic caudal to the third ramus communicans gives an after-discharge along the third ramus, closely resembling that set up along the post-

ganglionic nerve. On the other hand, tetanic stimulation of the third ramus gives no after-discharge in the thoracic sympathetic immediately caudal to it.

(2) Tetanic stimulation of the thoracic sympathetic trunk, immediately below the stellate ganglion, evokes no after-discharge in the sympathetic trunk below the fifth ramus. A stimulus applied to the sympathetic trunk, through these recording electrodes below the fifth ramus, sets up the discharge of a large spike (0·5 mV.) from the stellate ganglion along the cardiac nerves, i.e. a large number of preganglionic fibres ascend to the stellate ganglion from below the fifth ramus, and yet no after-discharge occurs back along these fibres when their repetitive stimulation gives a large and prolonged after-discharge along the cardiac nerves.

(3) When the cervical sympathetic is tetanized, large after-discharges are observed in the postganglionic trunk of the superior cervical ganglion, but no trace can be detected in the cervical sympathetic.

It therefore appears that the after-discharges observed in the third ramus are occurring in postganglionic fibres from the stellate ganglion. Such fibres have been described anatomically, and may be demonstrated physiologically. For example, when the thoracic sympathetic is stimulated caudal to the third ramus, a spike is observed in the third ramus, after the delay which would be expected for transmission to and from the stellate ganglion and synaptic relay there. It may therefore be concluded that after-discharge even from a deeply eserinized sympathetic ganglion occurs only along the postganglionic fibres. There is no discharge corresponding to the impulses backfired up the motor nerve fibres when eserinized muscle has been activated by their tetanization [Masland & Wigton, 1940; Eccles *et al.* 1942]. Correspondingly, acetylcholine sets up the discharge of impulses from the nerve terminals back along the motor nerve fibres [Masland & Wigton, 1940], but no such discharge is observed in the preganglionic nerve fibres [Bronk, 1939, Fig. 11].

Fig. 14*d* shows that successive doses of curare progressively diminish the intensity of the after-discharge from the fully eserinized ganglion, but its duration is little affected—for example, there is practically no falling off in its intensity during the 1·3 sec. of the record.

DISCUSSION

(*a*) *The synaptic potential of the curarized ganglion*

Eserine has little or no action on the time course of the synaptic potential and on the size of the second response at various intervals after the first (§ A1); hence summation of two synaptic potentials at different intervals and the time course of facilitation [cf. Eccles, 1943, § 5*c*] should not be appreciably affected by eserine. This has been confirmed with two experiments on the curarized stellate ganglion. A similar absence of effect has already been described for the action of eserine on the time course of facilitation with submaximal responses of the superior cervical ganglion [Eccles, 1935*b*, Text-fig. 9], and, since it indicated that cholinesterase did not play a significant role in the removal of the synaptic transmitter, it was regarded as evidence against the acetylcholine hypothesis of synaptic transmission.

There is convincing evidence that the synaptic potential is a catelectrotonic potential passively decaying in its latter part, and on this basis it has been analysed according to Hill's 'local potential' theory [1936] in order to obtain the probable time course of the active depolarizing agent produced by the preganglionic volley, i.e. of the 'transmitter-action' [Eccles, 1943]. The electric time constant is determined from the late passive part of the decay of the synaptic potential, set up either by a single preganglionic volley or by slow repetitive stimulation. A similar analysis may be applied to the eserinized synaptic potentials. Since the decay of synaptic potentials set up by a single volley or by slow repetitive stimulation has been affected little or not at all by eserine, it has been assumed that the electric time constant has been unaltered by eserine. The possible errors in the analysis have been discussed and shown not to be serious [Eccles, 1943]. A similar analysis of the end-plate potential gives a time course for the transmitter action in good agreement with more direct determinations [Eccles *et al.* 1941; Kuffler, 1942; Katz, 1942].

The single synaptic potentials shown in Fig. 1 were not appreciably altered by even deep eserinization, so, correspondingly, there is no alteration in the time course of the transmitter action. It shows characteristically the large initial 'peak' passing over to the small, more slowly decaying, 'tail' [Eccles, 1943, Fig. 7]. However, in the plotted curves of the transmitter action in Fig. 2 eserine has increased and prolonged the 'tail', but has had no appreciable effect on the time course of the initial 'peak'. In no experiment has eserine been observed to modify the time course of the rise and decay of the 'peak' of the transmitter action, but in several an effect on the 'tail' smaller than that of Fig. 2 has probably occurred. It was this absence of an eserine effect on the initial 'peak' of the transmitter action which formed the basis of the criticism of the acetylcholine hypothesis (cf. the indirect determination of the 'peak' action—which is there virtually the detonator response—in Eccles [1937*b*, Fig. 8]; also Eccles [1936*b*, pp. 367–70, 1937*c*]).

Analysis of the decaying phases of the repetitive responses of Fig. 6*a* gives the courses of transmitter action shown in Fig. 6*c*. With such rapid stimulation the decay of the non-eserinized transmitter action shows no initial peak, just a fairly slow decay for about 0·3 sec. [cf. Eccles, 1943, Fig. 8*d*]. The two eserinized responses (broken lines) show a similar fairly slow decay of the transmitter action passing over, at about 0·3 sec., to a level from which the decay is so slow that it can be entirely accounted for by the time constant of the amplifier. The brief initial 'peak', a small phase of intermediate rate resembling the 'tail', and the final stage of steadily maintained transmitter action may be recognized in the analysis of the response at 37 per sec. (Figs. 5 (iii) and 6*c*, dotted curve). In the responses of Figs. 3 and 4, analysed in Fig. 15, there are also various combinations of the initial 'peak', the intermediate stage resembling the 'tail' [cf. Eccles, 1943, Fig. 8] and the final stage of very slowly decaying transmitter action. Again, the time courses of the 'peak' and 'tail' are not appreciably affected by eserine. Thus the effect produced by all doses of eserine up to 10 mg./kg. consists chiefly, and probably entirely, in the addition of a new prolonged component to the transmitter action.

(b) *The synaptic potential of the normal ganglion*

As shown above (§ B2) there is good evidence that the *N* wave set up by a single preganglionic volley is largely composed of a negative potential, homologous with the synaptic potential of the curarized ganglion. There is thus an exact analogy with the end-plate potential at the neuro-muscular junction, where (as indicated by antidromic experiments [Kuffler, 1942]), after firing off the muscle spike and being suppressed thereby, the end-plate potential is again built up by the continuing transmitter action. In the single nerve-muscle-fibre preparation this building up only begins late on the declining phase of the spike; with the multifibre preparation, on the other hand, the end-plate potential was observed simultaneously with the spike summit [Eccles & Kuffler, 1941; Eccles *et al.* 1941, Fig. 12], an effect presumably attributable to the asynchronism of the individual spike responses. Similarly, in the ganglion, though the *N* wave increases the spike height [Eccles, 1935*c*; Lloyd, 1939], it probably resembles the end-plate potential in being suppressed until late on the declining phases of the spikes of the individual ganglion cells, for in such a fundamental reaction nerve and muscle presumably would behave similarly (cf. the corresponding impedance changes [Cole & Curtis, 1939; Katz, 1942]). The duration of the transmitter action observed in the curarized preparation ('peak' action of at least 10 msec. followed by a 'tail' up to 50 msec. duration [Eccles, 1943]), would be adequate to account for the building up of the synaptic potential after its suppression by the spike. On account of its long time-course the 'tail' action would sum during repetitive stimulation, and so be almost exclusively concerned in producing the large and prolonged synaptic potentials observed after rapid repetitive stimulation.

The synaptic potentials following repetitive preganglionic stimulations of varying frequency and duration also correspond closely with the effects observed for the synaptic potentials, with similar stimulations of the curarized ganglion. Assuming that the synaptic potential is suppressed during each spike potential, then the synaptic potential observed at the end of a tetanic stimulation must have been built up, after the last spike, by the surviving transmitter action. The time courses of the transmitter actions, following various tetani of the curarized ganglia, are shown typically in Fig. 15 [cf. Eccles, 1943, Fig. 8]. In every respect there is good agreement between the summed 'tails' of these transmitter actions in the curarized ganglia and the synaptic potentials observed at the ends of corresponding tetani of normal ganglia.

(i) With slow frequencies, e.g. 12 per sec., both the transmitter action and the synaptic potential (Fig. 7 (i)) differ but little from the single response.

(ii) As the rate is increased, the 'tail' of the transmitter action is larger and more prolonged, and *pari passu* the synaptic potential is larger and more prolonged (Figs. 7 (iii) and (ii), 9*c*).

(iii) As the duration at any frequency is increased (no durations longer than 0·7 sec. investigated) the 'tail' of the transmitter action is larger and more prolonged, and *pari passu* the synaptic potential is larger and more prolonged (Figs. 8, 9*a*, *b*).

(iv) The faster the rate (within limits—about 90–130 per sec. is the optimum) of a given number of stimuli, the larger the 'tail' of the transmitter action, and the larger the synaptic potential (Fig. 9*c*).

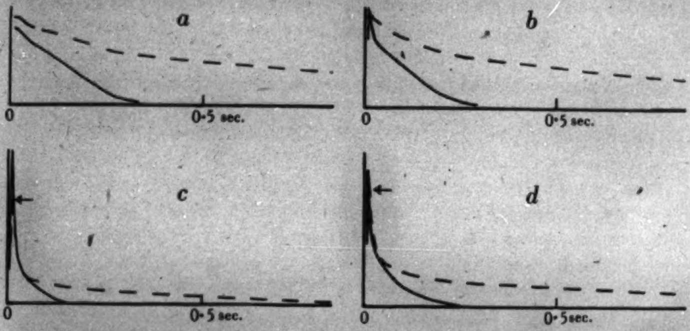

Fig. 15. Transmitter-action curves for potentials plotted in Fig. 4. Zero time corresponds to last stimulus of the repetitive series. *a*, 140 per sec. for 0·7 sec.; *b*, 84 per sec. for 0·7 sec.; *c*, 28 per sec. for 0·7 sec.; *d*, 55 per sec. for 0·35 sec. Arbitrary scale for ordinates.

The small change produced by eserine in the synaptic potential, normally set up by a single nerve volley (Fig. 8*a*), corresponds well with the small change in the curarized synaptic potential (Figs. 1, 2). However, with slow repetitive stimulation eserine produces a large increase in the normal synaptic potential (cf. Fig. 13*b*, 12·5 per sec. for 0·8 sec.), and yet no appreciable action on the curarized synaptic potential (Fig. 3 (v), 13·5 per sec. for 0·7 sec.). With the neuro-muscular junction, also, the eserine action was much more striking on the normal than on the curarized potentials [cf. Eccles *et al.* 1942, Figs. 4, 5].

The changes often observed in the concurrent *P* wave prevent an accurate determination of the extent to which eserine lengthens the synaptic potential (but see Fig. 8*d*). In those experiments, however, where the eserinized synaptic potential has been so large that it has submerged the *P* wave, it has persisted for seconds. Hence it is possible that, as with the curarized responses, eserine produces its effect on the synaptic potential of normal ganglia solely by adding a prolonged component, not normally present. The prolonged after-discharges from all eserinized ganglia (§ C3) provide additional evidence that the duration of the synaptic potential is prolonged to several seconds.

(c) *The spike response of the ganglion*

As the stimulation frequency is raised the brief initial peak of transmitter action in the curarized ganglion becomes smaller and eventually disappears (cf. Fig. 15*c*, *b*, *a*). With frequencies over 100 per sec. there would also be some fusion of the 'peaks' produced by successive impulses. Thus, up to about 100 per sec.—even higher rates for very short intervals [cf. Eccles, 1943, Fig. 8*d*]—the transmitter action would show a separate 'peak' for every stimulus, and the slower the rate the higher the 'peak'. There is therefore a remarkable correspondence between, on the one hand, the stimulation frequency and this peaked transmitter-action curve of the curarized ganglion, and on the other hand, the following of stimulation frequency by normal ganglionic discharge (Figs. 7, 12). There would seem to be no doubt that a ganglion can discharge at the stimulation frequency only so long as the transmitter-action curve shows sufficiently large and discrete 'peaks' at this frequency. Moreover, eserine has but little effect on the 'peaks' of the curarized transmitter-action curve, at least for short tetani (Fig. 15), and correspondingly it has no appreciable action on the transmission through the ganglion of the stimulation frequency (§ C1, Fig. 12).

(d) *After-discharge*

In the normal ganglion, after-discharge occurs after tetani have built up a large synaptic potential (§ B3), which, as we have seen, is due to the transmitter action accumulated by the rapid repetitive stimulation. The frequent appearance of after-discharges when the ganglion remains relatively positive during the summed synaptic potential may be taken to indicate that the after-discharge is not simply set up as a consequence of depolarization of the whole surface of the ganglion cells. If the depolarization only occurred in focal areas, the integrated potential that is recorded by the present methods could be positive. Again, it seems unlikely that the very prolonged after-discharges following prolonged stimulation [27 sec. in Bronk, 1939, Fig. 7], could be set up merely as a consequence of a maintained catelectrotonus, for there is a rapid accommodation to the synaptic potential [cf. Eccles, 1943, Fig. 5*b*]. Presumably, such long after-discharges are due to a prolonged negative potential set up by the continued action of some liberated substance, e.g. acetylcholine or K ions [cf. Bronk, 1939].

The striking effect of eserine in increasing and lengthening the after-discharge, and in causing it to appear with relatively slow rates of stimulation (e.g. 12 per sec.), obviously is related to the great lengthening of transmitter action observed with the synaptic potentials—both may persist for as long as 10 sec. after repetitive stimulation for less than 1 sec.

(e) *The transmitter action and acetylcholine*

In general these experiments have given striking support to the hypothesis dividing the transmitter action into short and long components of different type [Eccles, 1937 *c*, *d*]. The long transmitter action would include both the 'tail' action and the very prolonged action found in the eserinized ganglion.

There can be no doubt that the very prolonged transmitter action which appears in the eserinized ganglion is due to acetylcholine which is rapidly destroyed by cholinesterase in the non-eserinized ganglion. After a short preganglionic tetanus this prolonged action of acetylcholine (for several seconds) is observed equally well in the curarized ganglion as a synaptic potential, and in the normal ganglion as an after-discharge—and sometimes as a prolonged negative potential. It is to be noted that Lorente de Nó [1938] and MacIntosh [1938*a*] only occasionally succeeded in demonstrating the liberation of just detectable amounts of acetylcholine from the activated and fully eserinized superior cervical ganglion when it was perfused with blood. The present results give evidence for this liberation in every experiment. Further, the maximum action appears to be attained within a small fraction of a second after the last preganglionic impulse, there being no evidence for a delayed production [cf. Lorente de Nó, 1938]. Again, there is no sign that with repetitive stimulation the concentration of acetylcholine reaches a level at which it exerts a paralytic action, a complication which was suggested by Brown [1937*b*] and Rosenblueth & Simeone [1938*b*], even for the response to a single preganglionic volley.

The prolonged synaptic potential is observed after a very few stimuli (5 in Fig. 6*b*; and possibly even one in Figs. 2 and 8*a*), but the after-discharge has never been observed with fewer than 14 stimuli. This discrepancy is to be expected, because acetylcholine has to attain a threshold concentration in order to set up a discharge of impulses from the ganglion cell or the motor end-plate [Feldberg & Vartiainen, 1934; Govaerts, 1936; Brown, Dale & Feldberg, 1936; Brown, 1937*a*; Kuffler, 1943*a*], whereas no threshold concentration is observable for its depolarizing action at the motor end-plate [Kuffler, 1943*a*]. When a steady concentration of acetylcholine is perfused through the stellate ganglion, Bronk [1939] has observed a discharge of impulses continuing for minutes with no sign of accommodation; hence the prolonged after-discharges would be set up by acetylcholine regardless of the rapid accommodation of the ganglion to a continued catelectrotonus. It would seem, therefore, that the prolonged depolarization set up by persisting acetylcholine differs from that occurring at the cathode of a constant current.

This difference is probably attributable to a direct action of acetylcholine on the surface membrane, for example, lowering its impedance [cf. Katz, 1942], the local depolarization and the consequent discharge of impulses being both secondary to this action.

Eserine has no appreciable action on the 'peak' fraction of the transmitter action in the normal ganglion (§ C1), but the *P* wave prevents discrimination between any action it might have on the 'tail' and the above prolonged transmitter action. It will be assumed henceforth in the discussion that, as with the curarized ganglion, eserine has no appreciable action on the time course of either the 'peak' or the 'tail' fractions of the transmitter action (Figs. 6*c*, 15). In other respects, however, the 'peak' and the 'tail' fractions stand in sharp contrast.

(i) The duration of the 'peak' is brief (summit in 2·0–3·5 msec., total duration about 10–18 msec.) and is not appreciably altered by repetitive stimulation; the duration of the 'tail' is longer, about 50 msec. for a single response, and it is greatly lengthened by stimulation—up to at least 0·3 sec. after 100 stimuli at 140 per sec.

(ii) The 'peak' is largest with single stimuli, or stimuli at very low frequencies (10 per sec. or less); the 'tail' is smallest after single stimuli or stimuli at very low frequencies.

(iii) With faster rates of stimulation the 'peak' rapidly declines after the first few stimuli (it is usually largest with the second), and with rapid stimulation it eventually becomes very small (Fig. 15*b*) or even unrecognizable (Figs. 6*c*, 15*a*); the 'tail' builds up with rapid stimulation and within limits (cf. Fig. 15*a*, *b*) is larger the faster and more prolonged the stimulation.

Thus, with repetitive stimulation, the 'tail' transmitter action shows just that summation, increasing both intensity and duration, that would be expected for a transmitter substance liberated by the preganglionic terminals; on the other hand, the 'peak' transmitter action shows just the reverse behaviour.

In fact, the 'peak' action corresponds closely with the action which would be exerted if the preganglionic impulses also acted as electrical stimuli of the ganglion cells. Thus, under all conditions, the duration of the 'peak' action is sufficiently brief, for temporal dispersion of the incident preganglionic impulses would make the aggregate action considerably longer than the action current of a single preganglionic impulse. Again, the falling off in size of the 'peak' action with rapid repetitive stimulation would be partly due to the smaller size of the preganglionic impulses and, partly, to the background of depolarization produced by the summed action of the preceding impulses. In this connexion it is important to realize that the 'peak' action is measured solely by its effectiveness in producing additional depolarization. That, in the central nervous system, some mechanism of synaptic transmission exists other than acetylcholine has been indicated by the finding of MacIntosh [1941] that the concentration of extractable acetylcholine is negligibly small in some areas of the grey matter.

The action of curare in diminishing the 'peak' action may be regarded as evidence against its electrical genesis, for such doses of curare would have little or no action on the electrical excitability and response [cf. Fromherz, 1933]. However, it has now been shown that the specific action of curare in diminishing the acetylcholine sensitivity of muscle is exerted only on the acetylcholine sensitive end-plate region [Kuffler, 1943*a*]. The electrical excitability of this region or of the synaptic regions of ganglion cells has not yet been tested before and after curarization.

Evidence against electrical transmission was recorded by Coppée & Bacq [1938], who stated that during preganglionic degeneration, synaptic transmission failed when acetylcholine had largely disappeared from the ganglion [MacIntosh, 1938*b*], but before failure of transmission in the preganglionic fibres. However, their Fig. 2C shows that synaptic transmission was still present, for the negative ganglionic potential they record (and attribute to the preganglionic volley) seems indubitably to be a synaptic potential. If that is so, the small preganglionic volley shown in their Fig. 2B produced a transmitter action, setting up a local synaptic potential of some ganglion cells, which was below threshold for setting up the discharge of impulses. Histological examination has shown that, during preganglionic degeneration, synaptic transmission fails when the preganglionic nerve terminals (*boutons terminaux*) show a complete break-up of their structure [Gibson, 1940]. It may be concluded that degeneration studies have not given significant support to either the electrical or chemical theories of synaptic transmission.

If the 'tail' action is due to acetylcholine, this acetylcholine must be removed by some eserine-resistant mechanism, for example, resynthesis to its precursor [MacIntosh, 1938*a*]. Further, in the non-eserinized ganglion cholinesterase must function at a different locus, otherwise after eserine inactivation of the cholinesterase this eserine-resistant mechanism would automatically take over the removal of all the acetylcholine, and no prolonged transmitter action would be observed. On that view, the cholinesterase must therefore be more peripherally located relative to the site of liberation and action of the acetylcholine, only destroying that acetylcholine which has diffused away (cf. the 'barrier action' of cholinesterase suggested by Feldberg & Vartiainen [1934], MacIntosh [1938*a*], Eccles *et al.* [1942]). This 'barrier action' of cholinesterase accords well with the effects of progressive eserine dosage (cf. Fig. 6*a*) in causing an increase in the prolonged action, rather than a progressive slowing of its time course: as the barrier of cholinesterase is progressively more inactivated, more acetylcholine passes the barrier, and then, having escaped, is able to persist without any appreciable further destruction by cholinesterase. Eserine was found to cause in the normal ganglion a prolonged synaptic potential with fewer stimuli and slower frequencies (§ C2) than in the curarized ganglion (§ A2). This effect of curare in counteracting the appearance of the prolonged transmitter action was very striking with neuromuscular transmission, and extensions of the hypothesis in regard to the 'barrier action' of cholinesterase were suggested in order to explain this and other features of curare-eserine antagonism [Eccles *et al.* 1942]. These extensions could also be applied to the ganglion.

In summary, the following hypothesis of synaptic transmission seems best in accordance with the experimental evidence, though many other possible explanations could be devised. A preganglionic impulse exerts a brief depolarizing action on the ganglion cell by the direct effect of its action current (the 'peak action'). Superimposed on this depolarizing action at the beginning, and long outlasting it, is the 'tail action' due to the liberated acetylcholine (and possibly other substances such as K ions, cf. Bronk [1939]). The acetylcholine is removed from the locus of action by some eserine-resistant mechanism such as resynthesis to its precursor, but also partly by diffusion to the surrounding barrier of cholinesterase, where it is rapidly hydrolysed. Eserine inactivates this barrier and the acetylcholine is then free to spread along the surface membrane and exert an action persisting for seconds. After-discharge, either in the normal or eserinized ganglion, is set up by repetitive preganglionic stimulation when the acetylcholine (or other exciting substance of the 'tail' action) accumulates above a threshold concentration.

SUMMARY

1. Eserine has little or no action on the synaptic potentials which single or double preganglionic volleys set up in the curarized stellate ganglion cells. The synaptic potentials produced by preganglionic tetani are only changed in one respect by eserine—the addition of a very prolonged potential built up during the stimulation, and, on cessation of stimulation long outlasting (by several seconds) the decaying phase of the normal synaptic potential, which can still be observed superimposed on it.

2. The effect of frequency and duration of preganglionic tetanization has been observed on the responses (spike and after-potential) of the normal ganglion. Comparison with ganglionic after-potentials set up by similar antidromic tetanization has shown that, with preganglionic stimulation, there is an additional negative wave (a synaptic potential) superimposed on the early part of the after-potential. A single preganglionic volley sets up a small synaptic potential, and, with slow rates of stimulation (about 10 per sec.), it remains small; but with rapid rates of stimulation (50–140 per sec.) it sums up to a large potential, being initially even larger than the positive after-potential in some experiments. In all experiments this summed synaptic

potential can be sufficiently large to set up after-discharges from the ganglion cells, which may persist for as long as 0·3 sec.

3. Eserine has no appreciable action on the ganglionic after-potentials set up by antidromic stimulation, but, as in the curarized ganglion, it greatly lengthens the superimposed synaptic potential set up by preganglionic stimulation. Hence, summation is observed with much slower rates of stimulation, and there is a greatly prolonged (10 sec. or more) and intensified after-discharge with even less than one second's tetanization.

4. Analysis of the synaptic potential, according to the local potential theory, shows that the eserine effect is due to the production of a prolonged transmitter action without modifying the 'peak' and 'tail' transmitter actions normally present. This prolonged action is undoubtedly due to acetylcholine which normally is rapidly removed by cholinesterase. The properties of the 'tail' action show it to be due to a substance liberated by preganglionic stimulation, but not removed by cholinesterase. On the other hand, the brief 'peak' action (10–18 msec.) accords well with the direct electrical excitatory effect produced by the action currents of the preganglionic impulses.

The author wishes to thank the National Health and Medical Research Council of Australia for equipping and maintaining the workshop in which most of the apparatus was made, and also Merck Inc., U.S.A., for kindly donating the very high potency curare that was used throughout this investigation.

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THE ACTION OF ADRENALINE ON TRANSMISSION IN THE SUPERIOR CERVICAL GANGLION

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(*Received* 5 *November* 1943)

The present investigation has its origin in work of recent years in which it was observed that adrenaline sensitizes structures normally stimulated by acetylcholine. In skeletal muscle adrenaline potentiates the action of acetylcholine [Dale & Gaddum, 1930; Bülbring & Burn, 1942*a*], and it improves the transmission of motor nerve impulses at the neuro-muscular junction [Orbeli, 1923; Bülbring & Burn, 1940]. In the spinal cord the discharge of motor impulses following an injection of acetylcholine is facilitated and almost dependent on the presence of adrenaline. This may be explained by a lowering of threshold for stimuli reaching the motor nerve cell, since a similar facilitating action of adrenaline was seen on the flexor reflex [Bülbring & Burn, 1941]. The third site of action investigated was the sympathetic ganglion. Though Marrazzi [1939] had observed only an inhibitory effect of adrenaline at sympathetic synapses, Bülbring & Burn [1942*b*] obtained evidence that adrenaline affected nervous transmission in sympathetic ganglia, according to the dose administered. In perfusion experiments in dogs, the concentration of adrenaline in the circulation supplying the lumbar sympathetic chain was changed, without changing it in the responding organs which were the vessels of the hindleg, and it was found that small doses of adrenaline augmented ganglionic transmission, whereas large amounts depressed it. Results consistent with this observation were obtained in the whole animal: the pressor effect produced by splanchnic stimulation as well as the pressor effect due to the ganglionic action of acetylcholine was modified by adrenaline.

The logical step after this evidence was to see what effect adrenaline would have on the isolated sympathetic ganglion. Kibjakow [1933] was the first to perfuse the superior cervical ganglion of the cat, and, in the course of his study concerning the humoral transmission of preganglionic impulses, he made the observation that reinjection of the perfusate, collected during a prolonged stimulation, lowered the threshold to stimulation. This lowering of the threshold could be produced, not only in the ganglion from which the perfusate had been collected, but also in the ganglion of the other side which had been at rest. The effect lasted for 20–30 min., after which time the threshold rose again to its normal value.

In 1932 Iwanow published a review on the chromaffine and interrenal system of man in which he mentioned nests of chromaffine cells in sympathetic ganglia, and expressed the possibility of 'a local secretion of some substance which might act on the sympathetic synapse as its own stimulant or tonic' (p. 158 translated). In 1890 Smirnow, studying sympathetic ganglia of Amphibia, had shown that such chromaffine cells are innervated by nerve fibres which were otherwise indistinguishable from those which run to sympathetic nerve cells lying with them. Kohn [1903] found chromaffine tissue in sympathetic ganglia, particularly frequently in cats. It was Stöhr who in 1939 emphasized once more the presence of such 'Nebenzellen' in autonomic ganglia in man, lying in a network of fine nerve fibres and apparently connected with the preganglionic fibres. Glees [1942] showed that similar round cells are present in the superior cervical ganglion of the cat, lying closely around the actual ganglion cells. That these small cells show a chromaffine reaction could be seen in histological preparations of several superior cervical ganglia of cats which Mr E. H. Leach very kindly did for me.

The experiments to be described are concerned with the action of adrenaline on the perfused superior cervical ganglion of the cat, stimulated either through its preganglionic nerve or by injecting acetylcholine. In view of Kibjakow's original observation and the histological evidence of chromaffine tissue in sympathetic ganglia, which might be of physiological importance, the perfusate from the ganglion was collected during stimulation and tested for adrenaline-like properties.

METHODS

Cats were anaesthetized with chloralose. One superior cervical ganglion was perfused with Locke's solution using a small pump described by MacIntosh [1938]. All blood vessels connecting the ganglion with the rest of the body were tied, and the only connexion with the cat was formed by the nerve fibres leaving the ganglion and containing the postganglionic fibres to the nictitating membrane, the contractions of which were recorded.

The cervical sympathetic was cut and stimulated, with a pair of shielded platinum electrodes, by condenser discharges of varying frequency from a neon lamp circuit.

All injections were made into the arterial cannula which was fitted with a small rubber cap like that used by Gaddum & Kwiatkowski [1938] in rabbit's ear perfusions: a small air bubble underneath the rubber cap serves as an air cushion, preventing any change in perfusion pressure while the injection is made.

For the experiments in which the venous perfusate from the ganglion was tested for adrenaline-like properties, the Locke's solution had to be slightly modified. It was found that, in spite of atropine, the perfusate collected during preganglionic stimulation had an inhibitory effect on the frog heart, and a leakage of potassium from the ganglion was thought to be the possible cause [Vogt, 1936]. The Locke's solution for the ganglion perfusion in these experiments was therefore prepared with only half the amount of KCl (0·2 g. in 1000 c.c.). The solution contained also only one-third of the usual amount of bicarbonate (0·15 g. $NaHCO_3$ in 1000 c.c.), to render it less alkaline and thus to preserve any adrenaline liberated from the ganglion.

For the estimation of adrenaline in the venous perfusate four different methods were employed:

(1) *The perfused frog heart.* A method similar to that described by Tiegs [1934] was used. One cannula was tied into the vena cava and another cannula into the left aorta, the right aorta being tied. Ringer's solution from a Mariotte bottle entered the heart under a constant pressure head, and the arterial outflow tube was fixed at a constant height of 4 cm. above the level of the heart. The apex of the heart was attached with a fine silk thread to a long, light straw lever writing on a smoked drum. The whole heart preparation was submerged in a dish with Ringer's solution just covering it, and the Mariotte bottle was kept at such a level that the output of the heart was one drop per beat. This position was maintained throughout, starting about 4 hr. before any samples were tested. Injections of 0·25 c.c. of perfusate or known adrenaline solutions were made close to the venous cannula through a rubber cap into a small air bubble, thus preventing pressure changes as described above. Atropine 1 in 100,000 was added to the perfusion fluid in order to prevent any depression of the heart by the liberation of acetylcholine from the ganglion.

(2) *The isolated pigeon's rectum.* As fresh material for a hen's rectal caecum preparation [Gaddum, Jang & Kwiatkowski, 1939] was unobtainable, pigeons were tried. The caecum in these birds is only a very small appendix on each side of the rectum, and about 5 cm. of the rectum itself was therefore suspended in an isolated organ bath of 7 c.c. capacity. Spontaneous movements were often very irregular, but with a good preparation a clearly graded response to different concentrations of adrenaline could be obtained. Atropine was given in order to avoid obscuring any relaxation produced by adrenaline with a contraction due to release of acetylcholine from the ganglion.

(3) *Fluorescence.* Gaddum & Schild [1934] have described a method for detecting adrenaline by using the bright apple-green fluorescence which appears during the first minute after adding to a given solution one-tenth of the volume of $5N$ NaOH. This method was found to be very sensitive. The difference in appearance between a solution containing 1 in 1000 million adrenaline and one containing no adrenaline was quite distinct. Samples of perfusate from the ganglion were compared with known adrenaline solutions

made up in the same Locke's solution and ranging from 1 in 100 to 1 in 1000 million.

(4) *Shaw's colorimetric method* for estimating adrenaline [1938] was not sufficiently sensitive for quantitative assay. The qualitative 'specific test' was used in the earlier experiments, but the method was not used in the later ones.

RESULTS

The action of adrenaline on the effect of submaximal stimuli

When the cervical sympathetic nerve was stimulated with constant submaximal stimuli for 10 sec. at intervals of 2 or 3 min. the nictitating membrane gave a series of contractions of equal size. These contractions were increased by injecting a small dose of adrenaline into the perfusion fluid, while they were decreased by larger doses. Several factors influenced the result.

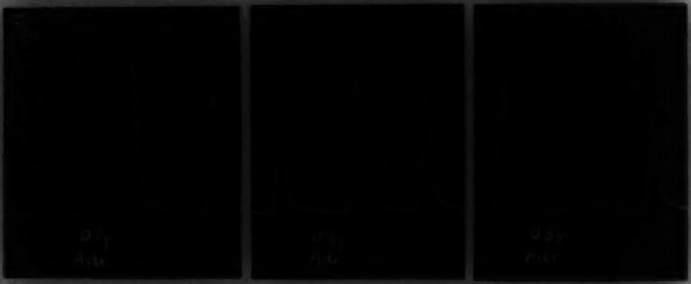

Fig. 1. Cat. Chloralose. Perfusion of superior cervical ganglion. Record of contractions of nictitating membrane in response to submaximal preganglionic stimulation, for 10 sec. at 2 per sec. Note augmentation by small dose and depression by large dose of adrenaline. For details see text.

Usually, doses of 0·1 μg. adrenaline or less produced an augmentation and larger doses a depression. The largest dose which increased the effect of stimulation was 0·3 μg. adrenaline; on the other hand, a dose as small as 0·01 μg. adrenaline once caused a depression which was, however, followed by an augmentation. These variations in sensitivity appeared to be due to differences in the rate of flow, which varied from 0·6 to 1·5 c.c. per min. in different ganglion perfusions. Thus, a given dose of adrenaline was comparatively large for one preparation and small for another. The effect of three different doses of adrenaline is illustrated in the experiment shown in Fig. 1. The cervical sympathetic nerve was stimulated at 3 min. intervals. In each section of the tracing one contraction before, one contraction 5 min. after the dose of adrenaline and one contraction 12 min. later are shown. An augmentation followed $0{\cdot}1\,\mu g.$ adrenaline, a smaller effect was seen after $0{\cdot}3\,\mu g.$, and a depression was caused by $0{\cdot}5\,\mu g.$ adrenaline.

The augmentor effect of adrenaline was obtained with slow rates of stimulation only. When the nerve was stimulated more than 8 times per sec. small doses of adrenaline had no effect, and depression was the sole result of larger doses. With stimulation at rates of 1, 2 or 6 per sec., doses of $0{\cdot}01$–$0{\cdot}05\,\mu g.$ adrenaline readily increased the contractions of the nictitating membrane.

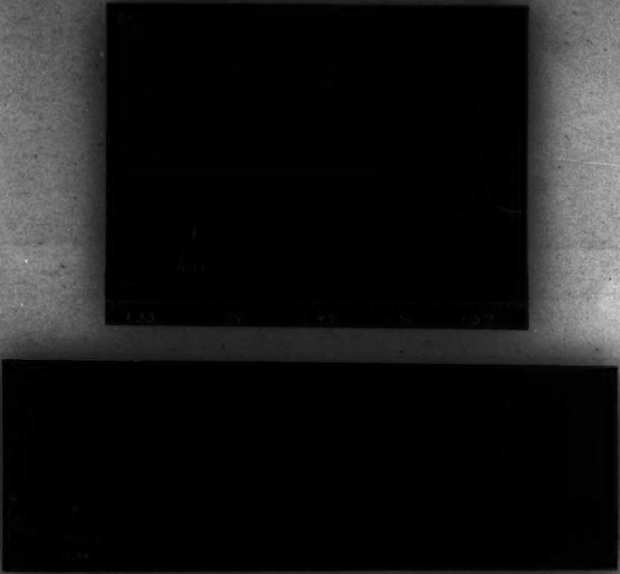

Fig. 2. Preparation as Fig. 1. *Upper tracing* shows every other contraction of nictitating membrane to 10 sec. stimulation at 2 per sec. every 3 min. Time given underneath. The augmentation produced by adrenaline lasted 20 min. *Lower tracing* shows contractions caused by stimulation at 2 min. intervals. An initial depression is followed, after 7 min., by prolonged augmentation (8 min. interval between last two contractions).

In Fig. 2 two tracings are given to illustrate the great variation from experiment to experiment in the response to adrenaline. The rate of stimulation was 2 per sec. in both. In one experiment $0{\cdot}1\,\mu g.$ adrenaline caused an augmentation which lasted for 20 min. In the other a 10 times smaller dose, $0{\cdot}01\,\mu g.$, caused a transitory depression followed by an augmentation which appeared

7 min. after the injection of adrenaline, and had not quite passed off 14 min. later.

The vasoconstriction produced by adrenaline had usually subsided before the contractions of the nictitating membrane returned to their original size.

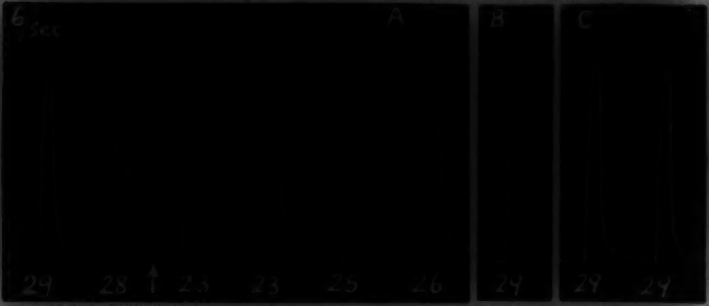

Fig. 3. Preparation as Fig. 1. Stimulation at 6 per sec. every 2 min. Figures are venous outflow drops per min. At arrow 0·01μg. adrenaline. Between (A) and (B) 4 min., between (B) and (C) 14 min. Note that the increase of contractions outlasts vasoconstriction.

Fig. 4. Preparation as Fig. 1. 10 sec. preganglionic stimulation at 6 per sec. every 3 min. Prolonged augmentation of response caused by perfusing the ganglion for 13 min. with Locke's solution containing adrenaline 1 in 200 million (A) before, (B) during, (C) after adrenaline perfusion.

In Fig. 3 is shown the effect of 0·01μg. adrenaline on transmission of submaximal stimuli at a rate of 6 per sec. The flow was reduced from 29 to 23 drops per min. When the flow had returned to normal the effect of stimulation was still increased, the increase only disappearing 24 min. after the administration of adrenaline.

The addition of adrenaline to the perfusion fluid in a concentration of 1 in 100 to 1 in 200 million had effects similar to those obtained by single injections. Fig. 4 shows the increased contractions of the nictitating membrane when adrenaline, 1 in 200 million, was added to the perfusion fluid. This was allowed to pass through the ganglion for 13 min. The potentiation of the stimulating effect, however, outlasted the adrenaline perfusion for over 20 min.

Fig. 5. Preparation as Fig. 1. Response of nictitating membrane to acetylcholine injected into the ganglion circuit. Prolonged augmentation produced by adrenaline; times since injection given underneath.

When perfusion of the ganglion with Locke's solution, containing adrenaline, was continued for 20 min. or longer augmentation was followed by depression, the effect of stimulation declined, and no recovery could be observed when perfusion was continued without adrenaline. When, moreover, adrenaline was added in higher concentrations than 1 in 100 million, the response to stimulation was depressed either without or after a very short augmentation.

Fig. 6. Preparation as Fig. 1. Twofold effect of adrenaline on the response of the nictitating membrane to acetylcholine injected into the ganglion circuit. See text.

The action of adrenaline on the response to acetylcholine

The contractions of the nictitating membrane in response to injections of acetylcholine into the ganglion circuit were influenced by adrenaline in the same way and by doses of the same order as those which modified the effects of preganglionic nerve stimulation. Thus, Fig. 5 shows the augmentation by 0·05 μg. adrenaline of the response to 10 μg. acetylcholine. The effect lasted for 20 min., rapidly passing off during the last 4 min. In Fig. 6 a larger dose of adrenaline, after a transitory augmentation, depressed the effect of acetylcholine; the contractions of the nictitating membrane returned to their former size within 15 min.

Changes in perfusion pressure from 100 to 150 mm. Hg did not change the size of the contractions elicited by acetylcholine, but they altered the duration of each contraction. Since, with the lower pressure, the flow was slower, the response to a given dose of acetylcholine was prolonged but not decreased in amplitude.

The action of pituitary posterior lobe extract on the effect of preganglionic nerve stimulation and of acetylcholine

In order to see whether the vasoconstrictor action of adrenaline was responsible for the facilitation of ganglionic transmission, the action of pituitary posterior lobe extract was studied. Pituitary extract had a strong vasoconstrictor action on the vessels of the ganglion. After doses of 0·001 unit, or more, the flow gradually stopped, and preganglionic stimulation became ineffective. Therefore, doses sufficiently small to cause a short lasting vasoconstriction only were chosen. They invariably depressed the effects of preganglionic stimulation and of acetylcholine. No dose of pituitary was found which increased transmission. In the experiment of Fig. 7 the effect of 0·0004 unit pituitary posterior lobe extract was compared with the effect of 0·01 μg. adrenaline. Adrenaline caused at first a 60% then a 30% reduction in flow with an increase in the contractions of the nictitating membrane. After the flow had returned to normal pituitary extract was injected. The

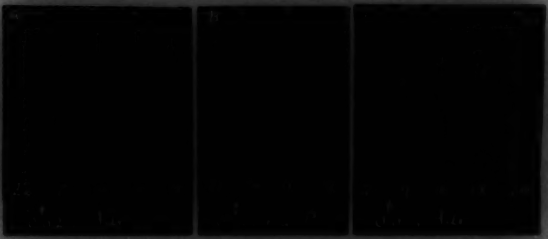

Fig. 7. Preparation as Fig. 1. 10 sec. preganglionic stimulation at 6 per sec. every 3 min. Figures are venous outflow drops per min. Between (A) and (B) 18 min., between (B) and (C) 15 min. The augmentation produced by adrenaline is shown in (A) and (C), the depression caused by pituitary (posterior lobe) extract is seen in (B).

flow was again reduced by 30%, but the contractions decreased in size and continued to decline while the vasoconstriction disappeared. A second dose of adrenaline produced a partial recovery.

Adrenaline-like properties of the venous perfusate from the ganglion

In these experiments the preganglionic nerve was stimulated maximally at a rate of 6 or 8 per sec. for 10–15 min. During this prolonged stimulation either very little or no reduction in flow was observed; in one experiment the flow became faster during stimulation.

Venous perfusate was collected before, during and after stimulation. Samples were tested by different methods for their adrenaline-like activity by comparing their effects with those of known solutions of adrenaline.

Fig. 8. Perfused frog heart. Assay of ganglion perfusate. C = injection of 0·25 c.c. perfusate collected before stimulation; S_7', after 7 min. stimulation; C_1', C_5' and C_{12}', 1, 5 and 12 min. after end of stimulation.

Nine experiments were performed in this way. In two experiments the nictitating membrane did not maintain its contraction during stimulation. The perfusate of one of these showed no activity by any of the four tests, and the perfusate of the other failed to stimulate the frog heart, although a trace of activity was found on the pigeon's rectum; it also showed some fluorescence of an intensity between that of an adrenaline solution 1 in 800 and 1 in 1000 million. Such a figure was sometimes found in control samples.

In six of the remaining seven experiments samples were assayed on the frog heart, comparing them with adrenaline. In all these experiments the samples collected during preganglionic stimulation stimulated the frog heart. In five of these experiments the fluorescence test was applied. It was positive, and, when compared with adrenaline, the concentration was found to agree with that found on the frog heart. In the same five experiments the perfusates were tested on the pigeon's rectum: no effect could be observed in two of them, the samples of three experiments caused relaxation, and, when compared with adrenaline, the results agreed with those obtained by the other methods. The colorimetric test (qualitative only) was twice positive, once negative, and twice not performed.

Fig. 9. Perfused frog heart. Assay of ganglion perfusate. C=injection of sample collected before stimulation; S_3', after 3 min. stimulation; C_5', 5 min. after end of stimulation.

Fig. 8 shows the effect of the perfusate on the perfused frog heart. The sample collected before stimulation only slightly affected the heart beat. The sample collected during the seventh minute of stimulation produced an effect similar in size to that of adrenaline 1 in 100 million but more prolonged. A sample collected 1 min. after the end of stimulation had still a strong effect. The activity disappeared gradually within 12 min. after the end of stimulation. The effect on the frog heart produced by ganglion perfusates was sometimes different in shape from that produced by adrenaline, but this was not always

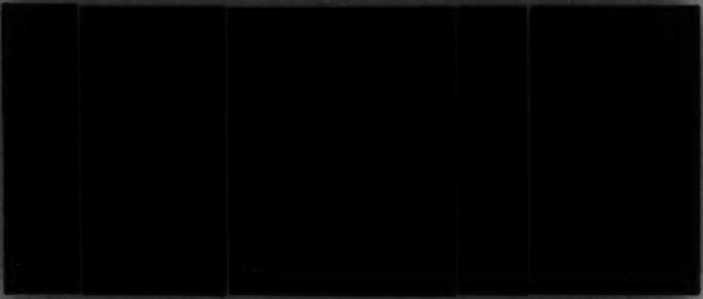

Fig. 10. Pigeon's isolated rectum. C=effects of 0·5 c.c. perfusate collected before and after stimulation; S, collected during stimulation, compared with adrenaline solutions.

so. For example, that shown in Fig. 9 closely resembles that produced by adrenaline being weaker than 1 in 100 million and stronger than 1 in 200 million.

Fig. 10 shows the effect on the pigeon's intestine produced by samples of the ganglion perfusate. All control samples caused a short relaxation, although the temperature of the freshly collected sample was about the same as that of the isolated organ bath. The inhibition caused by a sample collected during preganglionic stimulation, however, was prolonged and similar to that produced by adrenaline 1 in 200 million.

DISCUSSION

When the superior cervical ganglion of the cat is perfused with Locke's solution, the contractions of the nictitating membrane in response to a constant submaximal stimulus applied to the preganglionic fibres are increased by small doses of adrenaline, e.g. 0·01–0·1 μg. injected into the ganglion circuit, or by adding to the perfusion fluid adrenaline in a concentration of 1 in 100 to 1 in 200 million. Larger amounts of adrenaline depress ganglionic transmission. The increase is only observed when the stimulation is submaximal, and applied at a rate below 8 per sec. When the stimuli are maximal or at a faster rate, no increase is seen with small doses, and doses of more than 0·1 μg. diminish the contractions of the nictitating membrane. Similarly, the response to a constant dose of acetylcholine injected into the ganglion circuit is augmented by small doses of adrenaline, whereas larger amounts reduce the effect of acetylcholine.

Chromaffine tissue has been found histologically in the superior cervical ganglion of the cat, and it seems likely that a liberation of adrenaline inside the ganglion occurs simultaneously with the excitation of the synapse. The perfusate from the ganglion, collected during or immediately after preganglionic stimulation, contains a substance which stimulates the perfused frog heart, which relaxes the pigeon's isolated intestine, and shows fluorescence to the same degree as that produced by adrenaline in a concentration of 1 in 100 to 1 in 200 million. The fact that this concentration happens to be of the same order as that found to be the most effective in increasing the response of the nictitating membrane to submaximal preganglionic stimuli, supports the hypothesis that adrenaline plays an important part in synaptic transmission.

There are three different sources from which may arise the adrenaline appearing in the venous perfusate during preganglionic stimulation: the postganglionic neurones, the chromaffine tissue, or the sympathetic fibres terminating in the blood vessels of the ganglion. The last possibility is very unlikely because diminution in flow during a prolonged preganglionic stimulation was negligible or absent. There is already some evidence of the presence of an adrenaline-like substance in the superior cervical ganglia and other adrenergic

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THE PRESSOR BASES OF NORMAL URINE

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(*Received* 20 *November* 1943)

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A base which can be extracted from alkaline solution by toluene, and which gives an intensely yellow colour with picric acid in toluene-chloroform mixture, is found in the urine of dogs in which one kidney has been rendered ischaemic. This base is not found, except in traces, when fresh normal dog or human urine is similarly treated [Lockett & Verney, unpublished observations]. On steam distillation of normal human or dog urine from strongly alkaline solution, however, a similar base, to which I shall refer as the base B, has appeared in the distillate; and in an attempt to isolate the compound from which the base B is derived, a second base—base A—with powerful pressor activity, has been encountered. Both these bases are found in the steam distillate from dog and human urine made strongly alkaline with sodium hydroxide, and the experiments reported here have been selected to demonstrate their presence, to give some account of their properties, and to investigate their relationship to the pressor bases of normal urine previously reported in the literature.

EXPERIMENTAL

1. *The presence of two pressor bases, A and B, in the steam distillate from normal human urine made strongly alkaline with sodium hydroxide*

The chemical manipulations in the following experiment, which is representative of many others, are outlined in Scheme I.

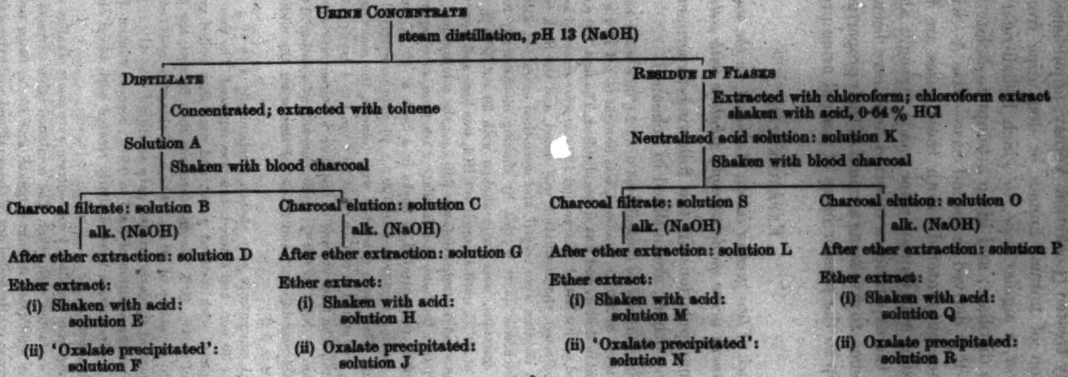
52 l. of normal human urine were concentrated to 12·2 l. on a water bath at 85° C. with a good draught. The concentrate, in 200 c.c. fractions, was brought to *p*H 13·0 with 10% NaOH, alizarin being used as external indicator, and was steam distilled. The first 120 c.c. of distillate from each fraction were reserved as distillate 1. The second 120 c.c. were reserved as distillate 2. When both fractions had been collected, the distillation was ended, and the flask residue was syphoned off into a pail. The flask residues were cooled, and were then extracted with 300 c.c. chloroform to 1·4 l. residue, in Winchester quart bottles, by shaking mechanically for ½ hr. The chloroform extracts, in 300 c.c. fractions, were twice shaken with 30 c.c. 0·64% HCl. The aqueous acid extracts were heated at 80° C. until all smell of $CHCl_3$ was lost, and were then brought just to the boil. The final volume after neutralization was 350 c.c.; the solution was green, and was reserved as solution K. Distillate 1 fractions and distillate 2 fractions were separately worked up. These distillates were gradually concentrated in flasks in a bath at 40° C., and at 5–11 mm. Hg, at such a rate that 4 l. were concentrated to 120 c.c. in approximately 10 hr. The concentrates were neutral to litmus. They were extracted with toluene, the aqueous phase was centrifuged and, after separation, was stored in the ice chest for from 1 to 4 days (distillate 1 concentrate and distillate 2 concentrate). The final volume of distillate 1 concentrate was 130 c.c.; and 45 c.c. of this, after the addition of NaCl to 0·9%, were reserved as solution A 1. The final volume of distillate 2 concentrate was 145 c.c.; and 50 c.c. of this, after the addition of NaCl to 0·9%, were reserved as solution A 2.

85 c.c. of distillate 1 concentrate were shaken mechanically with 10 g. blood charcoal (British Drug Houses Ltd.) for 30 min. and filtered: this was repeated. The first 10 g. of blood charcoal were washed at the pump with distilled water, and were then boiled gently, with stirring, for 10 min. with 80 c.c. of 0·64% HCl. The mixture was then filtered, the filtrate neutralized with NaOH and brought to a volume of 80 c.c.; 40 c.c. of this were reserved as solution C 1*a*. The second 10 g. blood charcoal similarly treated gave solution C 1*b*. 41 c.c. of the filtrate from the first 10 g. charcoal, after the addition of NaCl to 0·9%, formed solution B 1.

95 c.c. of distillate 2 concentrate were similarly treated with blood charcoal, solutions C 2*a*, C 2*b* and B 2 being obtained.

40 c.c. charcoal filtrate from distillate 1 concentrate were made alkaline with 4 c.c. 10% NaOH, and were three times extracted with ether. The combined ether extracts (30 c.c.) were divided, 15 c.c. being dried over anhydrous sodium sulphate, and 15 c.c. being twice shaken with 10 c.c. 0·64% HCl. The ether was gently boiled out of the combined acid extracts which, after neutraliza-

SCHEME I


URINE CONCENTRATE
steam distillation, pH 13 (NaOH)
DISTILLATE
Concentrated; extracted with toluene
Solution A
Shaken with blood charcoal
Charcoal filtrate: solution B
alk. (NaOH)
After ether extraction: solution D
Ether extract:
(i) Shaken with acid: solution E
(ii) 'Oxalate precipitated': solution F
Charcoal elution: solution C
alk. (NaOH)
After ether extraction: solution G
Ether extract:
(i) Shaken with acid: solution H
(ii) Oxalate precipitated: solution J
RESIDUE IN FLASKS
Extracted with chloroform; chloroform extract shaken with acid, 0·64 % HCl
Neutralized acid solution: solution K
Shaken with blood charcoal
Charcoal filtrate: solution S
alk. (NaOH)
After ether extraction: solution L
Ether extract:
(i) Shaken with acid: solution M
(ii) 'Oxalate precipitated': solution N
Charcoal elution: solution O
alk. (NaOH)
After ether extraction: solution P
Ether extract:
(i) Shaken with acid: solution Q
(ii) Oxalate precipitated: solution R

tion with NaOH to 21 c.c., formed solution E 1. The alkaline aqueous phase was neutralised with conc. HCl, the ether was boiled out, and the solution was reserved as D 1. To the dried ether extract was added a saturated solution of oxalic acid in ether, the precipitate was spun down, washed with ether, dried, and finally taken up in 10 c.c. 0·9 % NaCl: this formed solution F 1.

40 c.c. neutralized charcoal elution C 1 *a* were treated similarly, and solutions G 1, H 1 and J 1 were obtained corresponding with solutions D 1, E 1 and F 1 respectively.

250 c.c. solution K were twice shaken with 10 g. blood charcoal for 30 min.: the final filtrate was solution S. The first 10 g. blood charcoal, eluted by boiling and stirring with 250 c.c. 0·64 % HCl, filtered, neutralized with caustic soda, and brought to volume, gave solution O 1. The second 10 g. charcoal treated in the same way gave solution O 2.

184 c.c. solution S, to which 18 c.c. 10 % NaOH had been added, were three times extracted with ether. The combined ether extracts (70 c.c.) were divided. One portion of 35 c.c. was dried over anhydrous sodium sulphate, and to it was added a saturated solution of oxalic acid in ether; a very faint turbidity developed. The solution was centrifuged, the ether was decanted, the tube washed with ether on the centrifuge, the ether decanted, the tube dried, and finally washed with 20 c.c. 0·9 % NaCl: this formed solution N. The other portion of 35 c.c. was shaken with 20 c.c. 0·64 % HCl; this was repeated, and the ether was boiled out of the combined aqueous phases which, after neutralization with NaOH, formed solution M. The ether-extracted solution S, after neutralization with HCl, was boiled to dispel ether traces, and was diluted with water to 368 c.c.: this formed solution L.

200 c.c. of solution O 1 were treated in the same manner as solution S above, but 20 c.c. 10 % NaOH were used, and the final volume of solution P was 400 c.c. Solutions P, Q, and R corresponded with solutions L, M, and N.

The solutions, prepared as described above, were then tested for pressor activity on the anaesthetized cat. Anaesthesia was induced with chloroform and ether, and a 1·0 % solution of chloralose in 0·9 % NaCl was then injected intravenously in a dose of 0·08 g. chloralose/kg. body weight. The arterial pressure was recorded from the left carotid artery, clotting in the cannula being delayed by previously filling it with a solution of heparin (1 mg./c.c. of 0·9 % NaCl). A tracheal cannula was inserted; and the solutions, warmed to body temperature just before their administration, were injected by a syringe connected with a cannula which had been tied into the right femoral vein. The injection of each solution was immediately followed by that of 2 c.c. 0·9 % NaCl. In Table 1 are recorded the effects of these solutions on the arterial pressure.

The following control solutions were also prepared, and were found to be without action on the cat's blood pressure:

(1) 10 g. of blood charcoal eluted with 30 c.c. 0·64 % HCl, and the filtrate neutralized with NaOH. The amount injected intravenously was 3·0 c.c.

(2) 30 c.c. 0·64 % HCl shaken with ether, separated, the ether boiled out of the aqueous acid phase, and the solution, after neutralization with NaOH, brought to its original volume. 3·0 c.c. were injected intravenously.

(3) 20 c.c. of ether treated with a saturated solution of oxalic acid in ether, the solution centrifuged, the ether poured off, the tube dried, and then washed with 20 c.c. 0·9 % NaCl. 1·0 c.c. was injected intravenously.

TABLE 1

	Injected solution			Arterial pressure mm. Hg		
Cat no., wt., and date	Description	Dose c.c.	Original urine equivalent c.c.	Base line	Rise	Remarks
1, 2·2 kg., 3. x. 41	*A 1	2·0	800	150	86	—
	*B 1	2·0	800	142	22	—
	*C 1*a*	2·0	850	114	84	—
	C 1*b*	2·0	850	90	—	No response
	A 2	3·0	1080	92	72	—
	B 2	3·0	1080	103	12	—
	C 2*a*	3·0	1290	101	18	—
	C 2*b*	3·0	1290	103	—	No response
	A 2	3·0	1080	97	46	—
	A 1	2·0	800	95	80	—
	A 1	2·0	800	93	98	—
2, 2·6 kg., 3. x. 41	A 1	2·0	800	186	62	—
	D 1	2·0	728	187	—	No response
	E 1	2·0	760	187	—	No response
	F 1	3·0	2400	185	—	No response
	†A 1	2·0	800	184	59	—
	†C 1*a*	3·0	1275	180	61	—
	†G 1	3·0	1140	180	18	—
	†H 1	3·5	1417	182	53	—
	†J 1	3·0	2550	180	47	—
	‡K 1	2·0	300	178	47	—
	S 1	2·0	300	176	—	No response
	‡O 1	2·0	300	174	50	—
	‡P 1	4·0	300	173	23	—
	‡Q 1	1·0	375	170	40	—
	‡R 1	1·0	750	167	44	—
	N 1	1·0	690	167	—	No response
	K 1	2·0	300	167	53	—
	C 1*a*	3·0	1275	163	53	—
3, 2·2 kg., 4. x. 41	A 1	4·0	1600	159	44	—
	B 1	8·0	3200	157	23	—
	D 1	16·0	5824	147	28	—
	NaCl 0·9 %	16·0	—	137	18	—
	E 1	9·0	3420	133	8	No response
	NaCl 0·9 %	9·0	—	119	10	—

Control injections of 2·0 and 3·0 c.c. 0·9 % NaCl were made during the course of the experiments with cats 1 and 2: no significant change in blood pressure resulted.

* Responses illustrated in Fig. 1.
† Responses illustrated in Fig. 2*a*.
‡ Responses illustrated in Fig. 2*b*.

The blood-pressure responses of cat 1 to solutions A 1, B 1 and C 1*a* are given in Fig. 1, and those of cat 2 to solutions A 1, C 1*a*, G 1, H 1 and J 1 in Fig. 2*a*. From these results it will be seen that the greater part of the activity of the distillate concentrate was caused by a base which was adsorbed by blood charcoal, eluted therefrom by dilute acid, extracted from alkaline solution by ether, and which formed an oxalate insoluble in ether. The base of this oxalate will be referred to as the base A.

Since solution A 2 was active, but less so than A 1, it was probable that steam distillation had only partially removed the base from the urine concentrate. This is supported by the fact that solution K showed pressor activity caused by a base of similar character (Scheme I, Table 1 and Fig. 2*b*).

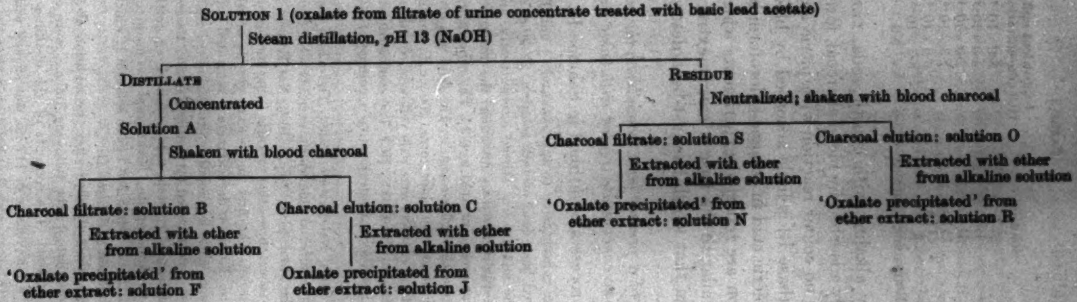
Fig. 1. Cat 1 (see Table 1). The tracings show the responses to the injection of the following solutions: *a*, 2 c.c. of A 1; *b*, 2 c.c. of B 1; *c*, 2 c.c. of C 1 *a*. *S* signals the times of injection. Time = 5 sec.

Fig. 2 *a*.

Fig. 2 *b*.

Fig. 2. *a* (above). Cat 2 (see Table 1). The tracings show the responses to the injection of the following solutions: *a*, 2 c.c. of A 1; *b*, 3 c.c. of C 1 *a*; *c*, 3 c.c. of G 1; *d*, 3·5 c.c. of H 1; *e*, 3 c.c. of J 1. Signal and time as in Fig. 1. *b* (below). Cat 2 (see Table 1). The tracings show the responses to the injection of the following solutions: *a*, 2 c.c. of K 1; *b*, 2 c.c. of O 1; *c*, 4 c.c. of P 1; *d*, 1 c.c. of Q 1; *e*, 1 c.c. of R 1. Signal and time as in Fig. 1.

SCHEME II


SOLUTION 1 (oxalate from filtrate of urine concentrate treated with basic lead acetate)
Steam distillation, pH 13 (NaOH)
DISTILLATE
Concentrated
Solution A
Shaken with blood charcoal
Charcoal filtrate: solution B
Extracted with ether from alkaline solution
'Oxalate precipitated' from ether extract: solution F
Charcoal elution: solution C
Extracted with ether from alkaline solution
Oxalate precipitated from ether extract: solution J
RESIDUE
Neutralized; shaken with blood charcoal
Charcoal filtrate: solution S
Extracted with ether from alkaline solution
'Oxalate precipitated' from ether extract: solution N
Charcoal elution: solution O
Extracted with ether from alkaline solution
'Oxalate precipitated' from ether extract: solution R

charcoal was eluted by boiling and stirring with 25 c.c. 0·64% HCl; the filtrate was neutralized and 6 c.c. were reserved as solution O.

85 c.c. of the charcoal filtrate derived from the flask residue after distillation were treated with 8·5 c.c. of 10% NaOH, and were extracted with ether; and the combined ether extracts were dried over anhydrous sodium sulphate, and were treated with a saturated solution of oxalic acid in ether. No visible precipitate formed. The ether was centrifuged, and decanted, and the tube was dried, and washed with 10 c.c. 0·9% NaCl, this giving solution N.

To 19 c.c. of the corresponding charcoal eluate were added 2 c.c. 10% NaOH, and this solution was similarly extracted, and the dried ether extracts treated with oxalic acid in ether. No turbidity resulted. The solution was centrifuged, the ether decanted, and the tube dried and then washed with 10 c.c. 0·9% NaCl: this formed solution R.

20 c.c. of distillate 1, to which 0·18 g. of NaCl had been added, were reserved as solution A 1. 80 c.c. of distillate 1 were shaken with blood charcoal, and 17 c.c. of the filtrate, with NaCl added to 0·9%, were reserved as solution B 1. 60 c.c. of the filtrate, treated with 6 c.c. 10% NaOH, were extracted with ether; the ether extracts were dried over anhydrous sodium sulphate, and were treated with a solution of oxalic acid in ether without visible effect. The ether solution was centrifuged and decanted, and the tube was dried and washed with 10 c.c. 0·9% NaCl: this formed solution F.

The charcoal was eluted with 25 c.c. 0·64% HCl, and the filtrate was neutralized with NaOH, 6 c.c. being reserved as solution C. 19 c.c. of the neutralized eluate were treated with 2 c.c. 10% NaOH, and were extracted with ether, the ether extracts being dried over anhydrous sodium sulphate. The addition of oxalic acid in ether caused a dense white turbidity, which spun down to a precipitate of very small amount. The ether was decanted, and the precipitate, after washing with ether on the centrifuge, was dried, and was taken up in 10 c.c. 0·9% NaCl: this formed solution J.

Distillate 2 was shaken with 3 g. blood charcoal, and the charcoal was eluted with 10 c.c. 0·64% HCl, and the eluate, after filtration and neutralization, gave solution A 2.

These solutions were tested for pressor activity on a cat weighing 2·5 kg. and prepared as previously described. Before injection, 3 c.c. of solution 1 were diluted to 15 c.c., 2 c.c. of solution C to 6 c.c., and 0·6 c.c. of solution J to 3 c.c. with 0·9% NaCl. The results are shown in Table 4, and the responses to solutions 1, A 1, A 2, C 1 and J 1 are illustrated in Fig. 3.

To 3 c.c. of each of the solutions were added 2 c.c. water and 0·5 c.c. 10% NaOH. Each was then extracted with 3 c.c. toluene, and the toluene extract was centrifuged. To 1·5 c.c. of each toluene extract 1·5 c.c. chloroform were added. On the addition of a drop of 2% picric acid in chloroform no colour appeared in any of the solutions.

The facts reported in this section show that a pressor base which is not precipitated by basic lead acetate solution is present in human urine. The base can be extracted from alkaline solution by ether and chloroform, and forms an oxalate which is insoluble in ether. The crude oxalate in a dose of 0·16 mg. (solution 1, Table 4, Fig. 3*a*) produced a rise of 64 mm. Hg in the arterial pressure of a 2·5 kg. cat under chloralose anaesthesia. The base is volatile in steam from alkaline solution, is adsorbed by blood charcoal from neutral solution, and gives no intensely yellow colour with picric acid in toluene-chloroform mixture. The properties of this base make it probable that it is identical with the base A described in the previous section. In a further

Moreover, the pressor activity of solution K was distillable in steam as the following procedures show.

60 c.c. of solution K, to which 5 c.c. 10% NaOH were added, were distilled in steam, the first 500 c.c. distillate being collected as D′ 1, the following 250 c.c. being collected as D′ 2. D′ 1 and D′ 2 were separately concentrated in the manner previously described, the volumes of concentrate being 73 and 33 c.c. respectively. To each of these concentrates NaCl was added to 0·9%. The flask residue was neutralized with hydrochloric acid, the final volume being 150 c.c., and to this 0·2 g. NaCl was added. 12 c.c. of solution K, and 14·8 c.c. of D′ 1 concentrate, were each diluted to 30 c.c. with 0·9% NaCl.

These diluted solutions, the neutralized flask residue, and the D′ 2 concentrate were then tested for pressor activity on a cat, wt. 2·2 kg., prepared as already described. The results are given in Table 2. It will be seen that the preparation of D′ 1 concentrate was associated with the loss of about one-third of the original pressor activity. The loss was attributed to the process of concentrating the distillate, and later experiments have confirmed the correctness of this view.

TABLE 2

	Injected solution			
Description	Dose c.c.	K equivalent c.c.	Original urine equivalent c.c.	Rise in arterial pressure mm. Hg
Diluted K	7·5	3·0	450	41
Flask residue	7·5	3·0	450	0
Diluted D′ 1	7·5	3·0	450	33
Diluted K	5·0	2·0	300	33
NaCl 0·9%	5·0	—	—	3
D′ 2 concentrate	7·5	13·6	2050	4

Now although the charcoal eluate C 1*b* (Scheme I and Table 1) was not active, a low-grade pressor activity remained in solutions B 1 and D 1. All the solutions given in Scheme I were then tested for the presence of a base extractable from alkaline solution by toluene and giving with picric acid an intense yellow colour in toluene-chloroform mixture. 1 c.c. of each solution together with 4 c.c. water and 0·5 c.c. 10% NaOH, were extracted with 3 c.c. toluene. After the toluene extracts had been spun, equal parts of toluene extract and chloroform, to which one drop of 2% picric acid in chloroform had been added, were matched in a colorimeter against a standard similarly prepared from a solution of known ephedrine hydrochloride content. This colorimetric test is a modification of that used by Richter [1938] in the estimation of ephedrine in urine. The colour production of each solution, expressed as equivalent ephedrine hydrochloride concentration, is given in Table 3. Were the low-grade pressor activity in solution B associated with this colour-producing base, we should expect that solution D 1 would show about the same activity when injected in double the dose. That this is so is seen in Table 1 (cat 3). There are present, then, in the steam distillate from human

TABLE 3

Solution	Equivalent ephedrine HCl concentration, g./c.c.
A 1	$8{\cdot}0\times10^{-4}$
B 1	$4{\cdot}0\times10^{-4}$
D 1	$2{\cdot}0\times10^{-4}$
C 1*a*	$7{\cdot}3\times10^{-5}$
C 1*b*	$6{\cdot}6\times10^{-5}$
K	$8{\cdot}3\times10^{-5}$
Remaining solutions	Nil

urine made strongly alkaline with caustic soda, two pressor bases, the one (base A) being readily adsorbed by blood charcoal, forming an oxalate insoluble in ether, and giving no colour reaction with picric acid in toluene-chloroform mixture; the other (base B) is little adsorbed by blood charcoal, and gives a strong colour with picric acid in toluene-chloroform mixture.

In the next section are reported experiments which have been selected to show that a base similar to the base A can be extracted from the urine by a process differing from that which has just been described.

2. *The extraction of a pressor base, resembling the base A, from the filtrate of urine-concentrate treated with basic lead acetate*

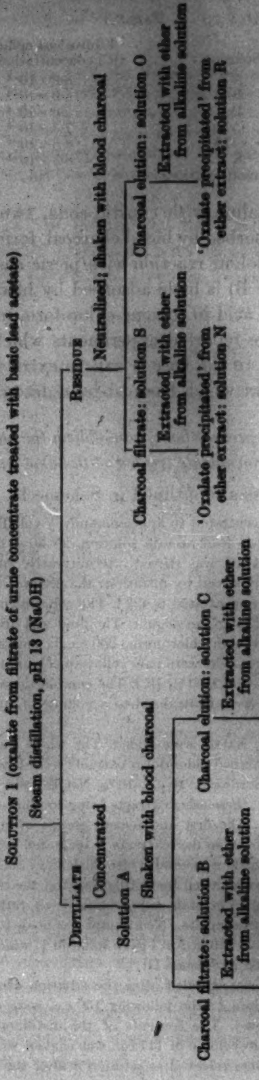
The steps in the process are outlined in Scheme II.

156 l. normal urine were concentrated to approximately ½ vol. The concentrate, after cooling, was treated with unfiltered basic lead acetate solution, 25–30 g. of solid being added for each litre of original urine. The mixture was stirred intermittently, and was then left to stand for 16–48 hr. The precipitate was removed by filtration; the filtrate, orange in colour, was freed of lead with H_2S gas, and was reconcentrated to 6·3 l. The concentrate was made strongly alkaline with NaOH, and was filtered through kieselguhr. The fluid was divided into three portions, and each was extracted three times with chloroform, 200 c.c./l., shaking mechanically for ½ hr. on each occasion. The chloroform extracts were pale yellow in colour, and, after having being centrifuged, were three times shaken with 0·64% HCl. The combined acid extracts were boiled gently to remove the chloroform, and were neutralized to a volume of 305 c.c. with 10% NaOH. This solution was pale yellow.

To this solution 30 c.c. 10% NaOH were added. The whole was three times extracted with 50 c.c. chloroform, and the combined chloroform extracts were twice shaken with 50 c.c. 0·64% HCl. After boiling out the chloroform, 20 c.c. 10% NaOH were added, and the solution was three times extracted with ether, these ether extracts being combined. Two subsequent extractions were kept in a separate group. The first and second groups of ether extracts were dried over anhydrous sodium sulphate. To these dry extracts a saturated solution of oxalic acid in ether was added. The first extracts gave a general white turbidity; the second extracts gave only a faint opalescence. The ether was removed by distillation, and the residue was recrystallized from alcohol: 10 mg. of waxy ill-formed crystals were obtained. A further 10 mg. were obtained by again working up the lead-free concentrate. No attempt was made to purify this oxalate. The total weight of solid dissolved in 25 c.c. 0·9% NaCl gave solution 1, which was treated as follows. The steps in the process are tabulated in Scheme II.

22 c.c. of solution 1 were steam distilled after the addition of 5 c.c. 10% NaOH. The first 100 c.c. were reserved as distillate 1; the following 150 c.c. were collected as distillate 2. The distillation was then discontinued. The contents of the distilling flask were neutralized with hydrochloric acid, and the whole volume of 117 c.c. was shaken with 3 g. of blood charcoal and filtered. 30 c.c. of the filtrate were reserved as solution S after the addition of 0·1 g. NaCl. The

SCHEME II


SOLUTION 1 (oxalate from filtrate of urine concentrate treated with basic lead acetate)
Steam distillation, pH 13 (NaOH)
DISTILLATE
Concentrated
Solution A
Shaken with blood charcoal
Charcoal filtrate: solution B
Extracted with ether from alkaline solution
'Oxalate precipitated' from ether extract: solution F
Charcoal elution: solution C
Extracted with ether from alkaline solution
Oxalate precipitated from ether extract: solution J
RESIDUE
Neutralized; shaken with blood charcoal
Charcoal filtrate: solution S
Extracted with ether from alkaline solution
'Oxalate precipitated' from ether extract: solution N
Charcoal elution: solution O
Extracted with ether from alkaline solution
'Oxalate precipitated' from ether extract: solution R

charcoal was eluted by boiling and stirring with 25 c.c. 0·64% HCl; the filtrate was neutralized and 6 c.c. were reserved as solution O.

85 c.c. of the charcoal filtrate derived from the flask residue after distillation were treated with 8·5 c.c. of 10% NaOH, and were extracted with ether; and the combined ether extracts were dried over anhydrous sodium sulphate, and were treated with a saturated solution of oxalic acid in ether. No visible precipitate formed. The ether was centrifuged, and decanted, and the tube was dried, and washed with 10 c.c. 0·9% NaCl, this giving solution N.

To 19 c.c. of the corresponding charcoal eluate were added 2 c.c. 10% NaOH, and this solution was similarly extracted, and the dried ether extracts treated with oxalic acid in ether. No turbidity resulted. The solution was centrifuged, the ether decanted, and the tube dried and then washed with 10 c.c. 0·9% NaCl: this formed solution R.

20 c.c. of distillate 1, to which 0·18 g. of NaCl had been added, were reserved as solution A 1. 80 c.c. of distillate 1 were shaken with blood charcoal, and 17 c.c. of the filtrate, with NaCl added to 0·9%, were reserved as solution B 1. 60 c.c. of the filtrate, treated with 6 c.c. 10% NaOH, were extracted with ether; the ether extracts were dried over anhydrous sodium sulphate, and were treated with a solution of oxalic acid in ether without visible effect. The ether solution was centrifuged and decanted, and the tube was dried and washed with 10 c.c. 0·9% NaCl: this formed solution F.

The charcoal was eluted with 25 c.c. 0·64% HCl, and the filtrate was neutralized with NaOH, 6 c.c. being reserved as solution C. 19 c.c. of the neutralized eluate were treated with 2 c.c. 10% NaOH, and were extracted with ether, the ether extracts being dried over anhydrous sodium sulphate. The addition of oxalic acid in ether caused a dense white turbidity, which spun down to a precipitate of very small amount. The ether was decanted, and the precipitate, after washing with ether on the centrifuge, was dried, and was taken up in 10 c.c. 0·9% NaCl: this formed solution J.

Distillate 2 was shaken with 3 g. blood charcoal, and the charcoal was eluted with 10 c.c. 0·64% HCl, and the eluate, after filtration and neutralization, gave solution A 2.

These solutions were tested for pressor activity on a cat weighing 2·5 kg. and prepared as previously described. Before injection, 3 c.c. of solution 1 were diluted to 15 c.c., 2 c.c. of solution C to 6 c.c., and 0·6 c.c. of solution J to 3 c.c. with 0·9% NaCl. The results are shown in Table 4, and the responses to solutions 1, A 1, A 2, C 1 and J 1 are illustrated in Fig. 3.

To 3 c.c. of each of the solutions were added 2 c.c. water and 0·5 c.c. 10% NaOH. Each was then extracted with 3 c.c. toluene, and the toluene extract was centrifuged. To 1·5 c.c. of each toluene extract 1·5 c.c. chloroform were added. On the addition of a drop of 2% picric acid in chloroform no colour appeared in any of the solutions.

The facts reported in this section show that a pressor base which is not precipitated by basic lead acetate solution is present in human urine. The base can be extracted from alkaline solution by ether and chloroform, and forms an oxalate which is insoluble in ether. The crude oxalate in a dose of 0·16 mg. (solution 1, Table 4, Fig. 3*a*) produced a rise of 64 mm. Hg in the arterial pressure of a 2·5 kg. cat under chloralose anaesthesia. The base is volatile in steam from alkaline solution, is adsorbed by blood charcoal from neutral solution, and gives no intensely yellow colour with picric acid in toluene-chloroform mixture. The properties of this base make it probable that it is identical with the base A described in the previous section. In a further experiment it has been found, as was to be expected from the pressor dose of the crude oxalate, that the base is very much more active than iso-amylamine.

TABLE 4

Injected solution			Arterial pressure mm. Hg		
Description	Dose c.c.	Original urine equivalent c.c.	Base line	Rise	Remarks
*1 (diluted)	1·0	1,250	159	64	—
*A 1	1·0	1,370	160	59	—
*A 2	1·0	13,800	160	26	—
B 1	1·0	1,560	164	—	No response
F 1	1·0	9,360	167	—	No response
*C 1 (diluted)	1·0	1,667	168	47	—
*J 1 (diluted)	1·0	1,900	164	48	—
S	1·0	1,340	162	—	No response
N	1·0	11,400	170	—	No response
O	1·0	6,240	171	—	No response
R	1·0	11,900	172	—	No response
1 (diluted)	1·0	1,250	170	44	—
A 1	1·0	1,370	162	48	—
1 (diluted)	1·0	1,250	161	46	—
A 2	1·0	13,800	160	21	—
C 1 (diluted)	1·0	1,667	163	42	—
J 1 (diluted)	1·0	1,900	164	34	—
1 (diluted)	1·0	1,250	160	46	—

Control injections of 5 c.c. 0·9 % NaCl, 1 c.c. of neutralized acid eluate of charcoal, and 1 c.c. of neutralized acid extract of ether (control of ether removal) were without effect on the blood pressure.

* These responses are illustrated in Fig. 3.

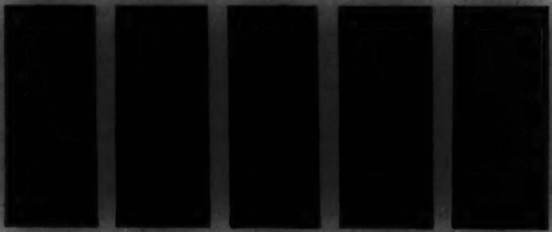


Fig. 3. The tracings show the responses to the injection of the following solutions: *a*, 1 c.c. of 1 (diluted); *b*, 1 c.c. of A 1; *c*, 1 c.c. of A 2; *d*, 1 c.c. of C 1 (diluted); *e*, 1 c.c. of J 1 (diluted). Signal and time as in Fig. 1.

3. *The precipitation of the pressor base found in the lead filtrate by mercuric chloride*

In order to discover whether or not loss of this base, whose isolation as an oxalate from the lead filtrate has just been described, occurs during the concentration of urine to a volume of one quarter of the original, and to relate the base to that described by Abelous & Bardier [1908], the following experiment is reported. The procedure followed that of these workers for urine: it was repeated on urine concentrate.

8 l. of normal human urine were collected in toluened receivers; the urine was well mixed, and was divided into eight separate fractions of 1 l. each. Two of these fractions were precipitated with basic lead acetate, being filtered after 21 and 41 hr. respectively. Two further 1 l. fractions were each treated with 20 g. mercuric chloride, in powder form, stirring being continuous for approximately 1 hr. during the addition of the powder. These fractions were filtered after 20½ and 42 hr. respectively. The excess lead or mercury from each fraction was removed by hydrogen sulphide, and, after filtration, each fraction was concentrated to a volume of 50–60 c.c. on a water bath.

The remaining four 1 l. fractions of urine were each concentrated to a volume of approximately 250 c.c. on a water bath at 80–85° C., with a good draught blowing over the surface of the fluid. Two of these fractions were precipitated with basic lead acetate, being filtered after 24 and 44 hr., and two were precipitated, each with 20 g. of $HgCl_2$, being filtered after 23½ and 45½ hr. respectively. The excess lead or mercury was removed from the filtrate by means of H_2S, and after filtration the fractions were concentrated to a volume of approximately 50 c.c.

Each fraction was made alkaline by the addition of powdered sodium bicarbonate, and wa then five times extracted with approximately 10 c.c. of ether. The ether extracts from each fraction were combined and were dried for 48 hr. over anhydrous sodium sulphate. The ether was then decanted, and was treated with a saturated solution of oxalic acid in ether until there was no further increase in opalescence. The ether solutions were centrifuged for 1 hr. The supernatant was decanted, and the residue was dissolved in 10 c.c. 0·9% NaCl.

These solutions were then tested for pressor activity on a cat weighing 3·0 kg. under chloralose anaesthesia, and prepared as previously described. The results are given in Table 5. The colorimetric test for the base B was negative on 0·5 c.c. of each of these solutions.

TABLE 5

Solution	Volume injected c.c.	Arterial pressure mm. Hg	
		Base line	Rise
From urine, precipitated with lead, 21 hr.	0·5	159	21
From urine, precipitated with $HgCl_2$, 20½ hr.	3·0	156	Nil
From urine, precipitated with $HgCl_2$, 42 hr.	3·0	156	Nil
From urine, precipitated with lead, 41 hr.	0·5	155	21
From urine concentrate, precipitated with $HgCl_2$, 23½ hr.	3·0	154	Nil
From urine concentrate, precipitated with lead, 24 hr.	0·5	155	18
From urine concentrate, precipitated with lead, 21 hr.	0·5	155	17
From urine concentrate, precipitated with lead, 21 hr.	0·5	153	20
From urine concentrate, precipitated with $HgCl_2$, 45½ hr.	3·0	153	Nil
From urine concentrate, precipitated with lead, 44 hr.	0·5	151	18

From these results it may be seen that there was no apparent loss of pressor activity by concentrating the urine to approximately a quarter of the original volume before precipitating with basic lead acetate, and that the same degree of pressor activity was obtained were the lead-treated urine or concentrate allowed to stand for one or two days. If, however, either urine or concentrate were treated with mercuric chloride, and allowed to stand for either one or two days, no pressor oxalate was obtained. Abelous & Bardier [1908] found that 'urohypertensin' was not precipitated by saturating urine with mercuric chloride.

4. *Preliminary adsorption experiments*

(*a*) *The adsorption of the bases A and B from the steam distillate by blood charcoal.* The former experiments, whilst clearly demonstrating the presence of the pressor base A, little more than indicate the presence of the base B. The presence of the latter base was more clearly shown in the course of a series of adsorption experiments which were carried out in an attempt to find a method of complete separation of the bases A and B which would allow of their simultaneous, almost quantitative, isolation from urine concentrate, and at the same time would afford information which would facilitate a search for these bases in tissue extracts.

The foregoing experiments show that, whereas the base A is rapidly adsorbed from neutral solution by blood charcoal, the base B is but little adsorbed. It was, however, found that, when fresh steam distillate was treated with blood charcoal, relatively large amounts of the base B were adsorbed. The steam distillate contains free ammonia which might alter the rate of adsorption of the bases A and B. The following experiment was designed to determine the rate at which the base A distilled in steam, and the effect of the high ammonia content of the steam distillate on the adsorption of the two bases by blood charcoal. Earlier experiments had suggested that the base A was adsorbed under these conditions more rapidly than the base B; eluates of blood charcoal which had been shaken with steam distillate had a high pressor activity, and contained both bases A and B. The base B could be roughly estimated as a colorimetric equivalent ephedrine hydrochloride concentration. Since the activity of the base B is low, and the dose of these charcoal eluates required to produce a pressor response was small, the pressor activity of these eluates was probably associated with the base A. To confirm this, it was planned to show the presence of the base A in the eluates by extracting the base from alkaline solution, subsequently precipitating the pressor oxalate, and to determine the total colorimetric equivalent, in terms of ephedrine hydrochloride, of a solution of the base B which was required to produce a pressor response. Such a solution of the base B could be obtained by concentration of the charcoal filtrate after all the base A had been adsorbed.

45 l. of normal human urine were concentrated on a water bath to 7·6 l.; the concentrate was distilled in steam at *p*H 13·0 (NaOH) in 200 c.c. fractions. The first 120 c.c. from each distillation formed the D 1 pool, the next 150 c.c. the D 2 pool. 500 c.c. of distillate from the D 1 pool were shaken with 0·5 g. blood charcoal for 20 min., and were filtered by suction: the charcoal was reserved as 1*a*. By shaking the filtrate with 0·5 g. blood charcoal and filtering, charcoal 1*b* was obtained. A repetition of the process gave charcoal 1*c*. 500 c.c. of distillate 2 similarly treated gave charcoals 2*a*, 2*b* and 2*c*. Each charcoal specimen was eluted with 20 c.c. 0·64% HCl by boiling gently and stirring, finally filtering at the pump. The filtrates were neutralized with NaOH, giving solutions which were numbered to correspond with the charcoals from which they were separated: solution 1*a*, 20·1 c.c.; 1*b*, 20·6 c.c.; 1*c*, 20·2 c.c.; 2*a*, 20·2 c.c.; 2*b*, 20·3 c.c.; and 2*c*, 20·1 c.c.

The ammonia content of 2 c.c. of each solution, and of the original distillates D 1 and D 2, was estimated by means of Nessler's reagent. Furthermore, 2 c.c. of each solution were diluted to 10 c.c. with water, and after the addition of 1 c.c. 10% NaOH were extracted with 3 c.c. toluene. The toluene was centrifuged. Equal parts of toluene extract and chloroform were treated with 1 drop 2% picric acid in chloroform, and the yellow solution so formed was compared in a colorimeter with a standard similarly prepared from a solution of known ephedrine hydrochloride content.

The neutralized solutions were tested for pressor activity on a cat weighing 2·0 kg. and prepared as previously described. The results are shown in Table 6. The colorimetric test was not applied to D 1 and D 2, since a high ammonia concentration in a solution causes turbidity in the final solution, making estimation impossible.

TABLE 6

Description of solution	Ammonia N mg./c.c.	Equivalent ephedrine hydrochloride concentration g./c.c.	Volume injected c.c.	Arterial pressure mm. Hg	
				Base line	Rise
D 1	1·10	—	—	—	—
D 1, after charcoal adsorption	0·77	—	—	—	—
1*a*	0·15	$6{\cdot}7 \times 10^{-4}$	0·5	128	33
1*b*	0·12	$1{\cdot}2 \times 10^{-4}$	7·0	132	26
1*c*	0·20	$3{\cdot}5 \times 10^{-4}$	7·0	136	23
D 2	0·75	—	—	—	—
D 2, after charcoal adsorption	0·63	—	—	—	—
2*a*	0·13	$2{\cdot}4 \times 10^{-4}$	3·0	133	45
2*b*	0·10	Trace	7·0	124	16
2*c*	0·11	Nil	10·0	132	13
1*a*	0·15	$6{\cdot}7 \times 10^{-4}$	1·0	130	44

Control injections 2·0, 5·0, 7·0 and 10·0 c.c. 0·9% NaCl, and 10 c.c. of blank charcoal neutralized solution had no appreciable effect on the blood-pressure trace.

These results show that the pressor responses resulting from the charcoal solutions are not paralleled by the colorimetric equivalent ephedrine hydrochloride concentrations. If the pressor response is due to the base A, the whole of the base A has not distilled over in the D 1 fraction, but is found also in the D 2 fraction. The adsorption of the base B simultaneously with the base A is seen, but occurs to a much greater degree in the D 1 fraction than in the D 2 fraction, suggesting that most of the base B has distilled over in the first fraction; however, the ammonia content of D 1 is considerably higher than that of D 2, and if the adsorption of the base B on blood charcoal is dependent on the presence of free ammonia, no conclusion can be drawn.

In order to confirm the suggestion that the pressor responses to the charcoal eluates described above were due to the base A, and to show that the amount of the base B contaminating the base A in these eluates was insufficient to cause any rise in blood pressure, the following procedure was adopted.

The combined solutions 1*a*, 1*b* and 1*c* were shaken with blood charcoal for 30 min., and the charcoal was eluted with 20 c.c. 0·64% HCl. The solution was made alkaline, and after filtering was extracted with ether. The ether extract was dried over anhydrous sodium sulphate, and was precipitated with a solution of oxalic acid in ether. The precipitate was spun down, dissolved in 20 c.c. 0·9% NaCl, and reserved as solution B.

The whole of the D 1 fraction remaining from the 45 l. of urine was now three times shaken with 0·75 g. of blood charcoal/250 c.c. of distillate. The whole weight of charcoal was eluted with 250 c.c. 0·64% HCl, this eluate being used for the adsorption experiments summarized below. 3·8 l. of the charcoal filtrate from D 1 were slowly concentrated as previously described. The final concentrate was three times shaken with 1·0 g. of blood charcoal, the last 1 g. being eluted with 20 c.c. 0·64% HCl, neutralized, and filtered; this solution was reserved as solution D. The charcoal filtrate from the final concentrate was filtered through kieselguhr, and NaCl was added to 0·9%, the total volume being 53 c.c. This solution was reserved as solution A. Colorimetrically the equivalent ephedrine hydrochloride concentration of this solution was $1{\cdot}6 \times 10^{-4}$ g./c.c.

These solutions, A, D and B, were tested for pressor activity on a cat weighing 1·3 kg. Solution D was without pressor action in a dose of 10 c.c., showing that the removal of the base A by means of blood charcoal was complete in solution A. 10 c.c. of solution A were required to produce a rise of 40 mm. Hg in arterial pressure; this dose would contain a total colorimetric equivalent of ephedrine hydrochloride of $1{\cdot}6 \times 10^{-3}$ g., and shows that the responses to the solutions shown in Table 6 cannot have been associated with the base B. Solution B, containing the precipitated oxalate from solutions 1*a*, 1*b*, 1*c* (Table 6), produced a supramaximal response in a dose of 1 c.c., confirming the supposition that the pressor activity of those solutions was dependent on their base A content; 0·15 c.c. of the solution B was negative to the colorimetric test for the base B.

(*b*) *A summary of the findings in further adsorption experiments with a number of different adsorbents.* The charcoal eluate, 250 c.c. in 0·64% HCl, from distillate 1, described above, was used for a series of adsorption experiments, the results of which are briefly summarized. This solution contained both bases A and B. The base A was followed by its pressor activity on intravenous injection into a cat under chloralose anaesthesia, and the base B was followed colorimetrically. In each case a knife point of adsorbent was added to 10 c.c. of solution.

Kieselguhr. No adsorption of either A or B from neutral solution, from a solution just alkaline to phenolphthalein with carbonate, or from a solution made strongly alkaline with caustic soda, or from weakly acid solution.

S.S.A.F.E. Fuller's Earth Company, Redhill, Surrey. Complete adsorption of A at neutral *p*H, and from weakly acid solution (mineral acid). Very slight adsorption of B from neutral solution, slight from weakly acid solution. Elution of A by 1% NaOH.

Kaolin. No adsorption of A or B from neutral solution, strong or weakly alkaline solution, or weak acid solution.

Lead sulphide. Slight adsorption of A only, and recovery by decomposition of the sulphide.

Animal charcoal. No adsorption of B or of A from neutral, or from alkaline solution.

Blood charcoal. From neutral solution, total adsorption of A, very slight of B. From a solution made just alkaline to phenolphthalein with sodium carbonate, very slight adsorption of B, total of A. Brought to the same *p*H with ammonia, considerable adsorption of B, total of A. From strongly alkaline solution (*p*H 13, NaOH), no adsorption of B, total adsorption of A. Elution occurred in all cases with dilute hydrochloric acid.

Permutit (Van Slyke). Adsorption of A from neutral and weakly acid solution, elution with alkali. No adsorption of B.

From the summary given above it is seen that a perfect separation of the two bases might be expected on Permutit for the estimation of ammonia as recommended by Van Slyke; its use on a large scale was, however, impracticable. Moderately good separation was to be expected from the use of blood charcoal (British Drug Houses, Ltd.) or S.S.A.F.E. (Fuller's Earth Company). These last were therefore tried in column adsorption experiments. An account of the charcoal adsorption experiments is given: the results obtained with S.S.A.F.E. were similar.

5. *Column adsorption experiments*

The following experiment clearly shows the zonal adsorption of the two bases A and B, which were further differentiated by means of simple chemical reactions, and provided material for a preliminary study of their absorption spectra in the ultra-violet.

(*a* 1) *The adsorption of the bases A and B in two zones on a blood charcoal column in the presence of added free ammonia.* 51 l. of urine were concentrated to approximately 11·5 l.; the concentrate was cooled and filtered, and was then distilled in steam in 200 c.c. fractions at *p*H 13 (NaOH), the first 120 c.c. of distillate being collected from each fraction. This distillate was shaken with 0·5 g. blood charcoal/250 c.c. The charcoal filtrate was slowly concentrated in the manner previously described, to a volume of 34 c.c. The charcoal was eluted by boiling and stirring with 65 c.c. 0·64% HCl, and was filtered off. The filtrate was neutralized with NaOH, and was filtered through kieselguhr. The neutralized eluate was added to the concentrated charcoal filtrate, and 6 c.c. of ammonia (sp. gr. 0·88) were added. A blood charcoal adsorption column was set up; a glass tube, length 15 in. and diameter 7 mm., was filled with blood charcoal, being connected above by thick rubber tubing to a funnel, and passing below into a receiver which was evacuated by means of a water pump. The solution was passed through in the course of 38 hr. Suction was kept up for 4 hr. after the last drop of solution had passed through.

The tube was then cut up into segments, which were numbered from the upper funnel end, and the charcoal content of each segment was eluted with 20 c.c. 0·64% HCl by heating in a boiling water bath for 20 min. with repeated shaking. After cooling, the suspended charcoal was spun down, the supernatant fluid was filtered by suction, was neutralized with 20% NaOH to litmus as external indicator, and was brought to volume.

The equivalent ephedrine hydrochloride concentration of these solutions was determined as before, and they were tested for pressor activity on a cat weighing 2·75 kg. and prepared in the manner previously described. The results are summarized in Table 7 A, and the responses to some of these solutions are shown in Fig. 4.

TABLE 7 A

Solution	Length of segment in.	Equivalent ephedrine hydrochloride concentration g./c.c.	Volume injected c.c.	Arterial pressure mm. Hg	
				Base line	Rise
1	½	$6{\cdot}0 \times 10^{-4}$	0·2	140	72
2	½	$6{\cdot}0 \times 10^{-4}$	0·2	140	53
3	½	$4{\cdot}8 \times 10^{-4}$	0·2	140	56
4	½	$1{\cdot}3 \times 10^{-3}$	0·1	138	65
5	1	$1{\cdot}4 \times 10^{-3}$	1·0	107	78
1	½	$6{\cdot}0 \times 10^{-4}$	0·2	107	73
6	1	$1{\cdot}4 \times 10^{-4}$	1·0	111	52
7	1	$1{\cdot}3 \times 10^{-3}$	1·0	117	44
8	1	$1{\cdot}2 \times 10^{-3}$	1·0	116	40
9	1	$1{\cdot}3 \times 10^{-3}$	1·0	110	36
10	1	$1{\cdot}3 \times 10^{-3}$	1·0	109	38
11	1	$1{\cdot}0 \times 10^{-3}$	1·0	109	27
Filtrate	—	$7{\cdot}5 \times 10^{-4}$	3·0	92	103

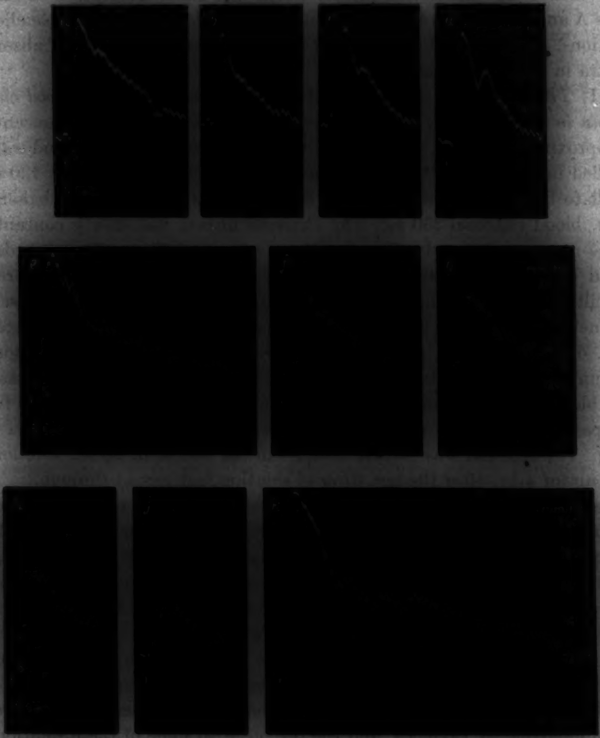


Fig. 4. The tracings show the responses to the following solutions: *a*, 0·2 c.c. of 1; *b*, 0·2 c.c. of 2; *c*, 0·2 c.c. of 3; *d*, 0·1 c.c. of 4; *e*, 1·0 c.c. of 5; *f*, 1·0 c.c. of 6; *g*, 1·0 c.c. of 7; *h*, 1·0 c.c. of 8; *j*, 1·0 c.c. of 11; *k*, 3·0 c.c. of the final filtrate. Time and signal as in Fig. 1.

From Table 7A and Fig. 4 it may be seen that the pressor activity of the neutralized charcoal eluates falls into two zones. The upper zone is of greater activity, and extends from segments 1–4 inclusive; the lower zone extends from segments 5–11 inclusive, and appears to be carried on into the filtrate. When the length of segment is considered, it is seen that the activity of solutions from the upper zone is twenty times as great as the activity of solutions from the lower zone If the activity of the lower zone be associated with a base giving an intensely yellow reaction in chloroform-toluene mixture, and roughly estimated as equivalent ephedrine hydrochloride concentration, this second base must be contaminating the upper zone, but in amount which could not then be responsible for the pressor activity of a dose of 0·1 c.c. For the further examination of these zones, typical solutions were selected, and their reactions to benzoylation, and to extraction with ether from alkaline solution followed by the precipitation of the dried ether extract with a saturated solution of anhydrous oxalic acid in ether, were studied.

(a 2) *The further differentiation of these zones by means of benzoylation and by extraction by ether from alkaline solution, with subsequent oxalate formation.* 1 c.c. of solution 2 was diluted to 3 c.c. with 0·9% NaCl. 3 c.c. of this dilution, and 3 c.c. of solutions 5, 7, and the final filtrate, were benzoylated as follows. 0·1 g. NaOH was added and dissolved. The alkaline solutions were cooled in ice. Six small drops of benzoyl chloride were added; after the addition of each drop the solution was vigorously shaken for 5 min., and was then again ice cooled. The reaction was at the end still faintly alkaline. 2·0 c.c. water were added, and the solutions were centrifuged for 40 min. The supernatant fluid was decanted, and was warmed on a water bath at 70° C. for 20 min. After cooling, the solution was made just acid to litmus with HCl, and was cooled in ice for 2 hr. The benzoic acid was spun down, and the supernatant fluid was neutralized with NaOH, and was brought to a volume of 9 c.c. with water. The NaCl content was approximately 1·5%.

1 c.c. of solution 2 diluted to 5 c.c. and 5 c.c. of solutions 5, 7 and 10, and of the final filtrate, were treated as follows. 0·1 g. NaOH was added and dissolved. Each alkaline solution was three times thoroughly extracted with ether, and the ether extracts were shortly dried, for 3 hr. only, over anhydrous sodium sulphate. The ether was decanted, and was treated with a slight excess of oxalic acid in ether. Any precipitate forming was spun down, the overlying ether solution was decanted, the tube was gently blown dry, and the precipitate was dissolved in 10 c.c. 0·9% NaCl.

The solutions were numbered to correspond with those from which they were prepared, but with the suffix B to denote benzoylation, and A to denote attempted oxalate formation.

The equivalent ephedrine hydrochloride concentration was determined on 2·0 c.c. of each of the benzoylated solutions and on 1·0 c.c. of each of the oxalate solutions. The solutions were tested for pressor activity on a cat weighing 2·2 kg. and prepared as previously described. The results are given in Table 7 B. This cat was very much less sensitive than the cat used on the previous day (Table 7 A), as is seen by the responses given to identical solutions.

TABLE 7 B

Solution	Volume injected c.c.	Equivalent volume of original solution c.c.	Equivalent ephedrine hydrochloride concentration g./c.c.	Arterial pressure mm. Hg Base line	Arterial pressure mm. Hg Rise
2	0·2	—	$6\cdot0 \times 10^{-4}$	132	21
2 A	1·0	0·1 of solution 2	Trace only	139	16
	2·0	0·2 of solution 2	—	143	22
5	1·0	—	$1\cdot4 \times 10^{-6}$	141	23
5 A	2·0	1·0 of solution 5	$3\cdot1 \times 10^{-4}$	141	17
7	1·0	—	$1\cdot3 \times 10^{-3}$	141	17
7 A	2·0	1·0 of solution 7	$2\cdot5 \times 10^{-4}$	135	6
10	1·0	—	$1\cdot3 \times 10^{-3}$	131	14
10 A	3·9	2·0 of solution 10	$3\cdot3 \times 10^{-4}$	130	19
Filtrate A	4·0	2·0 of filtrate	$1\cdot0 \times 10^{-4}$	123	Nil
Filtrate	3·0	—	$7\cdot5 \times 10^{-4}$	120	29
5 B	6·0	2·0 of solution 5	None	113	Nil
Filtrate B	6·0	2·0 of filtrate	None	105	Nil
2 B	6·0	0·66 of solution 2	Trace only	99	56
2	0·6	—	$6\cdot0 \times 10^{-4}$	112	44
7 B	6·0	2·0 of solution 7	None	111	Nil
1 K	0·2	0·2 of solution 2	—	132	19
1	0·2	—	—	132	21
1 K	0·4	0·4 of solution 2	—	133	33
1 KUV	0·4	0·4 of solution 2	—	135	34

(*a* 3) *Ultra-violet absorption of solutions from these two zones.* Solution 1 was eight times filtered through kieselguhr, and 2·8 c.c. of this solution were exposed to ultra-violet light during the taking of an absorption spectrum. The exposed and unexposed solutions were tested on the same cat, to determine whether such exposure caused any alteration in the pressor activity of the solution, and the results are shown in Table 7 B. The kieselguhr filtrate is lettered 1 K and the exposed solution 1 KUV.

The ultra-violet absorption spectrum of solution 1 K was studied, a control solution being prepared from a blank charcoal eluate prepared as was solution 1, and being similarly filtered through kieselguhr. A 1 in 20 dilution of 1 K (diluted with 0·9% NaCl) showed an absorption band at 2600 A. A solution prepared from the combined residual fluid of solutions 6–11 inclusive, and having an equivalent ephedrine hydrochloride concentration of $4\cdot5 \times 10^{-4}$ g./c.c., showed no absorption band. These solutions were tested for pressor activity at the end of the ultra-violet examination. The base B solution was tested on a cat weighing 1·8 kg., prepared as before. 3·0 c.c. produced a rise in blood pressure of 66 mm. Hg from a base line of 108 mm. Hg. 3·0 c.c. of the 1/20 dilution of 1 K gave a rise of 76 mm. Hg from a base line of 81 mm. Hg.

In this column experiment, evidence is again obtained of the presence of two pressor bases in the steam distillate from strongly alkaline urine concentrate. In a series of comparable eluates, the dose required to produce a comparable pressor response, when injected into a cat under chloralose anaesthesia, falls into two zones. The upper zone is short, and of great pressor activity; the lower zone is long, and has, for the same volume of charcoal, up to $\frac{1}{20}$th the activity of the first zone. The pressor activity of the second zone roughly parallels the approximate colorimetric equivalent ephedrine hydrochloride concentration of the solutions derived from it. This zone appears to extend into the final filtrate. The pressor compound in the first zone withstands benzoylation and readily gives an oxalate insoluble in ether. The pressor compound of the second zone is readily benzoylated, but is not readily extracted by ether from alkaline solution to give an oxalate insoluble in ether: the colour development with picric acid in toluene-chloroform mixture continues parallel to the pressor activity throughout the processes of benzoylation and oxalate formation.

(*b* 1) *A comparison of the zonal adsorption of the bases A and B on blood charcoal columns in the presence and absence of free ammonia.* In order to give more information about the effect of ammonia on the adsorption of these bases, and to show that there is no third base present in the final filtrate of the preceding experiment, the following experiment is recorded.

42 l. of normal human urine were concentrated to 8·2 l., and the concentrate was cooled and filtered, and was then steam distilled in 200 c.c. fractions at *p*H 13 (NaOH), the first 120 c.c. of distillate being collected from each fraction. The distillate was shaken with blood charcoal, 0·5 g./250 c.c. The charcoal filtrate was concentrated slowly, in the manner previously described, to a volume of 158 c.c. and was filtered through kieselguhr. The charcoal was eluted with 430 c.c. 0·64 % HCl, and after neutralization with NaOH was filtered through kieselguhr, the final volume being 442 c.c. The eluate and the concentrate were combined, and NaCl was added to 0·9 %. Three glass tubes, length 18 in., diameter 7 mm., were filled with blood charcoal, and the solution was slowly run through, the receiver being evacuated by a water pump. The process took 48–54 hr., the suction being kept up for 4–6 hr. after the last drop.

Tube 1. Blood charcoal. 90 c.c. of the fluid to which 4 c.c. conc. ammonia solution had been added.

Tube 2. Blood charcoal. 120 c.c. of the fluid to which 9 c.c. conc. ammonia solution had been added.

Tube 3. Blood charcoal. 100 c.c. of the fluid, without added ammonia.

After the fluid had passed through, the tubes were each separately cut up into small segments, the charcoal content of each segment being washed with 7–8 c.c. of water into a numbered centrifuge tube. 0·5 c.c. 13 % HCl were added, and the volume was brought to 10 c.c. The tubes were rolled, and were heated in a boiling water bath for 5 min., and were then cooled, and spun. The tube sides were washed down with 3 c.c. of water, and the spinning was repeated. The supernatant was filtered by suction, was neutralized to litmus paper with 20 % NaOH, and the volume was brought to 15 c.c., and the solution filtered. These latter filtrates were numbered according to their corresponding segments, from the funnel end downward.

0·5 c.c. of each solution was estimated for the colorimetric equivalent ephedrine hydrochloride concentration as previously described. Each solution was tested for pressor activity by intravenous injection into a cat under chloralose anaesthesia. Tubes 1 and 2 were tested on a cat weighing 2·2 kg., tube 3 on a cat weighing 3 kg. These cats were prepared in the manner previously described. The results are summarized in Table 8.

TABLE 8

Solution	Length of segment in.	Equivalent ephedrine-hydrochloride concentration g./c.c.	Volume injected c.c.	Arterial pressure mm. Hg	
				Base line	Rise
		Cat, 2·2 kg.			
Tube 1: 1	1	$5·3 \times 10^{-4}$	0·2	83	52
2	1	$6·2 \times 10^{-4}$	4·0	82	48
3	1	$5·2 \times 10^{-4}$	4·0	83	35
4	1	$5·3 \times 10^{-4}$	4·0	84	39
5	1	$5·3 \times 10^{-4}$	4·0	82	39
6	1	$3·35 \times 10^{-4}$	4·0	87	31
7	1	Trace	4·0	83	10
8	1	Negative	4·0	73	Nil
9	1·25	Negative	4·0	72	Nil
Filtrate	—	Negative	5·0	89	12
Tube 2: 1	1·25	$8·4 \times 10^{-4}$	0·1	134	50
2	0·75	$5·7 \times 10^{-4}$	4·0	129	35
3	1·0	$5·7 \times 10^{-4}$	4·0	133	50
4	1·25	$6·3 \times 10^{-4}$	4·0	127	59
5	0·75	$2·6 \times 10^{-4}$	4·0	121	30
6	1	Trace	4·0	106	Nil
7	1	Negative	4·0	106	Nil
8	1	Negative	4·0	97	Nil
9	0·5	Negative	4·0	89	Nil
Filtrate	—	Negative	5·0	95	39
0·9 % NaCl	—	—	4·0	83	Nil
		Cat, 3·0 kg.			
Tube 3: 1	0·5	$2·35 \times 10^{-4}$	2·0	88	143
2	1	$1·2 \times 10^{-4}$	5·0	86	Nil
3	1	$1·1 \times 10^{-4}$	—	—	—
4	1	$1·1 \times 10^{-4}$	—	—	—
5	1	$1·1 \times 10^{-4}$	—	—	—
6	1	$1·1 \times 10^{-4}$	—	—	—
7	1·5	$5·5 \times 10^{-5}$	—	—	—
8	1·5	Trace	—	—	—
Filtrate	—	$3·1 \times 10^{-4}$	10·0	91	113

In tubes 1 and 2 of this experiment, the eluates of the first segment show very strong pressor activity. The dose required of the charcoal eluate of the second segment to produce a comparable rise of blood pressure is at least twentyfold, and this range of dose is maintained through the segments until the end of the pressor eluates. The disappearance of the pressor effect synchronizes with the disappearance of the colour test associated with the base B. A low-grade pressor activity was found in the final filtrates from these solutions: these final filtrates before injection were neutralized with HCl, and were brought to theoretical volume, some evaporation having taken place. Their pressor effect was later shown to be due to the NH_4Cl they contained. 10 c.c. of a final filtrate, containing 2·2 mg. of ammonium nitrogen/c.c., gave a 30 mm. rise of blood pressure on intravenous injection into a cat under chloralose anaesthesia, this pressor response equating exactly with a similar solution of NH_4Cl in 10 c.c. 0·9% NaCl. Other final filtrates were similarly checked.

In the absence of added ammonia (tube 3), the adsorption of the first pressor substance, presumed to be the base A, proceeds in the first segment as before, but very little of the base B is adsorbed, the greater part passing into the final filtrate.

Eluates from a control blood charcoal tube treated with 0·9% NaCl, and from another treated with 0·9% NaCl containing ammonia, were without pressor activity.

(*b* 2) *The differentiation of these zones by benzoylation and by oxalate formation.* In order to differentiate these zones more completely, the following procedure was adopted.

The solutions of tube 1, 1–5, were benzoylated. To 10 c.c. of each solution 0·1 g. NaOH was added; and the solution was cooled in ice. Small drops of benzoyl chloride were added every 3 min., vigorous shaking being maintained. After the addition of 4–5 drops, the solution was just alkaline to litmus. It was centrifuged, the supernatant fluid decanted, and warmed on a water bath to destroy any residual benzoyl chloride. After it had been cooled and made just acid to litmus with HCl 5%, the solution was put in the ice chest for 2 hr., and was then filtered by suction. The filtrate was neutralized with NaOH, and diluted to 15 c.c. These solutions were numbered according to their original segments, but with the suffix B.

The solutions of tube 2, 1–5, were made alkaline by the addition of 0·1 g. NaOH. Each solution was three times extracted with 6 c.c. ether, and the ether extracts from each solution were separately dried for 12 hr. over anhydrous sodium sulphate. The ether was decanted, and was treated with a saturated solution of oxalic acid in ether. Any precipitate forming was spun down, the supernatant fluid was decanted, and the tube was drained and dried, the precipitate being taken up in 10 c.c. 0·9% NaCl. Such solutions were numbered according to their original column and segment, but with the suffix A.

These solutions were tested for pressor activity on cats prepared as previously described, and their colorimetric equivalent ephedrine hydrochloride concentrations were determined. The results are summarized in Table 9, and some of the responses are shown in Fig. 5. The results of this column experiment, therefore, confirm those of the last experiment, but show in addition that, whereas the base A of the upper zone is adsorbed in the presence or absence of free ammonia, the base B of the second pressor zone is adsorbed appreciably only when free ammonia has been added. The possible presence of a third pressor compound in the filtrate from the first experiment is excluded.

Both these experiments show the adsorption of two pressor bases, separated into two fairly well-marked zones. The upper zone is short, and of great activity. The lower zone is longer and of lower activity. The pressor activity of this zone is closely paralleled by the equivalent ephedrine hydrochloride concentrations of the solutions derived from it; and in the second column experiment the end of this zone coincides with the disappearance of the colour-producing compound. In each experiment the colour-producing compound is seen to extend upward throughout the first zone, but a comparison of the pressor dose with the colour equivalent shows that in this zone the pressor effect is not associated with the colour-producing compound; moreover, after benzoylation there is complete disappearance of both the colour-producing compound and the pressor activity from the second zone, but in the first zone there is almost complete disappearance of colour production without loss of pressor activity (Table 7 B). Extraction by ether from alkaline solution, followed by precipitation and resolution of the oxalate, is complete for pressor activity in the first zone, but very little colour-producing compound has survived this treatment; in the second zone the results are variable, but the pressor activity is again parallel to the intensity of colour production. An absorption band at 2600 A. is found in a solution from the first zone, but not in solutions from the second zone. No attempt was made in these experiments to correlate the height of this band with the pressor activity of solutions from the first zone, but it was shown that exposure to ultra-violet light in the process of taking an ultra-violet absorption spectrum did not alter the pressor activity of the solutions.

TABLE 9

Solution	Equivalent ephedrine hydrochloride concentration g./c.c.	Volume injected c.c.	Arterial pressure mm. Hg		Cat wt. kg.
			Base line	Rise	
Tube 1: 1 B	$6{\cdot}7\times10^{-5}$	7·5	128	114	3·0
2 B	Negative	10	119	Nil	—
3 B	Negative	10	117	Nil	—
4 B	Negative	10	108	Nil	—
5 B	Negative	10	121	Nil	—
Tube 2: 1 A	$1{\cdot}3\times10^{-4}$	0·5	122	58	—
		1·0	84	121	2·75
2 A	$1{\cdot}1\times10^{-4}$	8·0	80	Nil	—
3 A	$1{\cdot}1\times10^{-4}$	8·0	90	Nil	—
4 A	$1{\cdot}6\times10^{-4}$	8·0	56	Nil	—
5 A	Negative	8·0	61	Nil	—

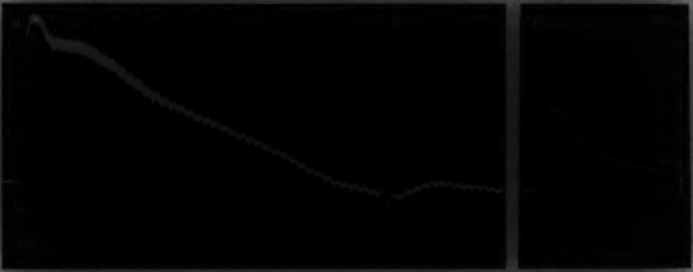


Fig. 5. The tracings show the responses to the injection of the following solutions: *a*, 7·5 c.c. of 1 B, tube 1; *b*, 10 c.c. of 2 B, tube 2; *c*, 0·5 c.c. of 1 A, tube 2. Time and signal as in Fig. 1.

Further experiments of this type showed that, were the steam distillate from alkaline urine concentrate treated with charcoal, in the manner previously described, columns through which the neutralized charcoal eluate with added ammonia had been passed showed the same two zones, but the second zone was short; were the concentrate of the charcoal filtrate alone passed through, a single zone, corresponding to the second zone, was found.

Throughout the above experiments urine was collected in toluened pails with lids; these pails were changed 2–3 times a day, and the urine was worked within 16 hr., or discarded, but no attempt was made to use only sterile urine. In order to determine whether or no the presence of these two bases could be attributed to bacterial action, the following experiment was performed.

6. *The presence of the two bases A and B in the steam distillate of urine made strongly alkaline with caustic soda, and under conditions ensuring the absence of bacterial activity*

Fresh normal human urine, passed between 8 a.m. and 9 a.m., was collected into clean heavily toluened pails. Evaporating pans, flasks, and all apparatus to be used were heated in a hot-air oven at 130° C. for not less than 30 min. immediately before use. A sample of the mixed urine was transferred by sterile pipette to a sterile flask. This sample was delivered at 9.30 a.m. to Dr E. F. Gale (Biochemical Laboratory, Cambridge), who very kindly cultured it for me. The cultures were made on a glucose casein-digest marmite medium, and were incubated at 37° C. Three experiments were carried out. No organisms grew from the urine sample in the second of these experiments, and this is the experiment reported.

The experiment was begun at 9.30 a.m. The urine was at once concentrated on a water bath at 85° C. When concentration was completed to approximately $\frac{1}{5}$ vol., the concentrate was at once steam distilled in 200 c.c. fractions at *p*H 13·0 (NaOH) without cooling or filtration. Six fractions were run simultaneously, 120 c.c. of distillate being collected from each 200 c.c. fraction, and the process took less than 2 hr. The distillate was collected in sterilized bottles containing blood charcoal, 0·5 g./250 c.c., and was mechanically shaken, and filtered. The filtrate was at once concentrated by the method previously described. The charcoal was immediately eluted by boiling with 0·64% HCl: the filtrate was neutralized and filtered, and was then shaken with 0·2 g. of fuller's earth (S.S.A.F.E.). The fuller's earth was eluted with aqueous alkali, the eluate was extracted with ether, and the ether extracts were dried overnight over anhydrous sodium sulphate. The ether was decanted next morning, a slight excess of a solution of oxalic acid in ether was added, and the precipitate was spun down, was washed with ether, and then was dissolved in 20 c.c. 0·9% NaCl. This solution was sterilized by gentle boiling, and was brought to volume only just before injections were made. To the charcoal-filtrate concentrate, NaCl was added to 0·9%. This solution was three times shaken with blood charcoal to ensure the complete removal of the base A, and was then sterilized by boiling, and was stored in the ice chest until the following morning. This process was completed soon after midnight. The cat was prepared at 9.30 a.m. the following day.

Both bases were found to be present in normal amount. From 10·3 l. of human urine 27 doses of the base A were obtained as the crude oxalate, a dose being that equating with 25 μg. of adrenaline tartrate, and 6 equi-pressor doses of the base B were obtained. The pressor activity of these solutions was determined on a cat weighing 2·2 kg. and prepared in the manner previously described.

DISCUSSION

Abelous & Bardier [1908] found in human urine a highly active pressor base which was not precipitated by basic lead acetate. This base could be extracted by ether from alkaline solution, and gave an oxalate insoluble in ether. Solutions of this base gave no colour reaction with ferric chloride. In all these points the 'urohypertensin' of Abelous & Bardier resembles the base A described in this paper. Abelous & Bardier found, however, that 'urohypertensin' was not precipitated when urine was saturated with mercuric chloride. In an experiment reported in this paper, the base A was completely precipitated by mercuric chloride. Abelous & Bardier state that 'urohypertensin' is not found in dog urine. In experiments to be reported in a later paper, the base A has been found in the urine of bitches in amount comparable with that in human urine. The base B, so far as I am aware, has not been detected before.

Bain [1915] reported the finding of isoamylamine in human urine. I have not yet been able to find isoamylamine in urine, be it fresh or stale, sterile or infected.

SUMMARY

1. Two pressor bases are shown to be present in the steam distillate from urine concentrate made alkaline with caustic soda.

2. Some account is given of the properties of these two bases.

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ACTION OF ACETYLCHOLINE, ATROPINE AND ESERINE ON THE CENTRAL NERVOUS SYSTEM OF THE DECEREBRATE CAT

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The investigations carried out in recent years by different groups of workers [Schweitzer & Wright, 1937 *b*, *c*; Schweitzer, Stedman & Wright, 1939; Kremer, Pearson & Wright, 1937; Kremer, 1942; Bonnet & Bremer, 1937; Torda, 1940; Bülbring & Burn, 1941] have proved conclusively that both acetylcholine and eserine have a direct action on the central nervous system in various species (man, dog, cat, frog, toad). There are, however, considerable differences in the results obtained in different species and on the different preparations employed. Thus, Schweitzer & Wright [1937 *b*, *c*], working on the knee jerk of cats under chloralose anaesthesia, found that acetylcholine produced generally a transient central inhibition (intensified by eserine), though sometimes an initial stimulation was observed. Bülbring & Burn [1941], working on the isolated perfused spinal cord of the dog, obtained with acetylcholine a motor discharge from the resting spinal cord; the knee jerk showed transient depression followed by stimulation, and the flexor reflex was potentiated. Kremer [1942] injected acetylcholine intrathecally in man in doses from 2 to 500 mg. without result, though, when the drug was administered together with subliminal doses of prostigmine, it depressed muscle tone and reflexes by a central action. Turning to eserine, Schweitzer & Wright [1937 *b*, *c*] found that the drug markedly increased the knee jerk and produced convulsions in chloralosed cats. Bülbring & Burn [1941], on the contrary, obtained with the isolated perfused spinal cord of the dog depression of the knee jerk, and potentiation of both the motor and inhibitory effects of acetylcholine. We have examined the actions of acetylcholine, atropine and eserine on the central nervous system in the decerebrate cat.

METHODS

The preliminary procedures were carried out under ether-chloroform anaesthesia. The sciatic nerves to both hindlimbs were cut high up to exclude central effects on the flexor muscles. Drills were passed into the upper and lower ends of both femurs. The left subclavian artery was isolated and its branches to the muscles of the forelimbs, the costocervical artery and thyrocervical axis were tied; the only branches left open were the internal mammary and vertebral arteries. Injections of acetylcholine were made into the central end of the subclavian artery so prepared; eserine was injected intravenously. In analysing the site of action of the drugs (whether central or peripheral) two procedures were employed: (*a*) the contralateral quadriceps muscle was denervated; (*b*) the hindlimbs were made ischaemic by clamping the abdominal aorta, usually after preliminary ligation of its branches below the renal arteries, as described by Schweitzer & Wright [1937*c*]. After completing these measures, the cat was decerebrated at the level of the anterior colliculi and allowed to rest for ½–1 hr., so that the anaesthetic might be eliminated. Tension changes in the quadriceps muscles were recorded by connecting the ankle by means of threads running over pulleys to Sherrington torsion lever myographs. The knee jerk was elicited by the electrically operated device of Schweitzer & Wright [1937*a*]. Acetylcholine solutions were made up in 5% NaH_2PO_4 and diluted before use in acid saline (pH 4–4·5). Control injections of the acid saline alone, either intra-arterially or intravenously, had no effect on the nervous reactions studied.

RESULTS

Action of acetylcholine on quadriceps muscle tone

Acetylcholine injected intra-arterially into the central end of the subclavian artery increases the tension of the unstimulated innervated quadriceps muscle (Figs. 1, 2). The dose producing the response is 25–100 μg. The effect comes on almost without detectable latent period; the tension rises abruptly to a degree which varies considerably in different preparations. The maximum response recorded showed a tension of 1·7 kg. above the base-line tension due to the decerebrate rigidity itself. The relaxation phase may be completed rapidly in 10–20 sec. or be more prolonged (40–60 sec., or even up to 120–150 sec.). There may be an initial phase of extensive rapid relaxation followed by a drawn-out phase in which the relaxation gradually becomes complete; or relaxation may proceed uniformly in step-like falls with intermediate short plateaux; or intermediate curves may be obtained (Fig. 1 A–D). No tension change whatsoever takes place in the denervated contralateral quadriceps. The rise of tension coincides with the initial rise of arterial blood pressure, which is mechanically produced by the injection of fluid into the circulation; the muscle-tension peak is attained before the fall of blood pressure due to the acetylcholine sets in. There is no relationship between the degree, duration or general pattern of the blood-pressure fall or the rate of recovery and the character of the relaxation curve of the quadriceps. Respiration is often stimulated, becoming deeper and faster [Schweitzer & Wright, 1937*b*], and over-ventilation may outlast the changes in the quadriceps; the respiratory response does not seem to influence the reaction of the quadriceps.

In addition to the response in the hindlimb, a widespread reaction is often observed, involving, in the forelimbs, increased extension at the elbow and abduction at the shoulders; the trunk may be thrust into opisthotonus, the animal taking up a posture of 'exaggerated' decerebrate rigidity.

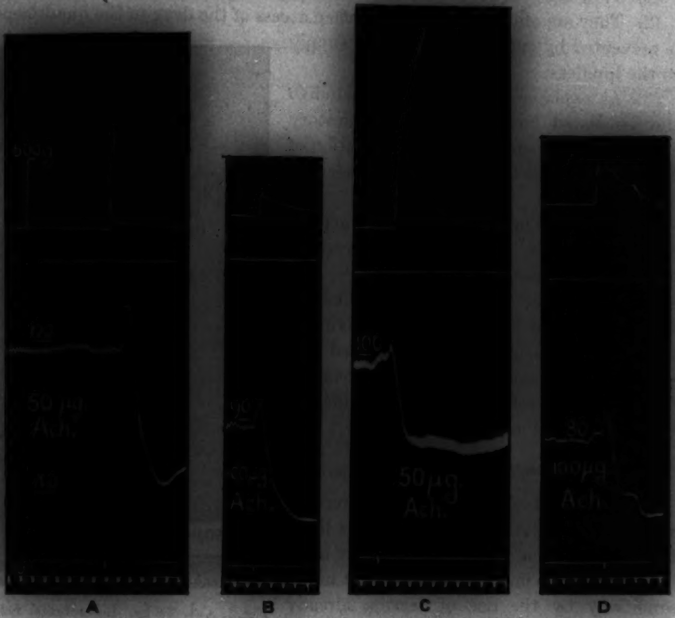

Fig. 1. Cat. Decerebrate. Sciatic nerves cut. Responses obtained in four different animals to injection of acetylcholine (50–100 μg.) into the central end of the subclavian artery. Records from above downwards: tension of innervated quadriceps; tension of denervated quadriceps; blood pressure, mm. Hg; signal; time 10 sec.

Repetition of the initial dose in the same animal may sometimes give comparable responses over a series of injections; in other preparations, however, there may be a progressive decline in the responses. The responses in the two limbs (if both are innervated) are not necessarily identical in magnitude, though similar in general form. The dose commonly producing a response was 50 μg. In about one-third of the experiments no positive reactions were obtained with doses up to 100 μg. We refrained from increasing the dose further, owing to the permanent fall of blood pressure and circulatory failure which results from such a large dose of acetylcholine, in the unatropinized animal. Equal doses of acetylcholine injected intravenously (into the jugular vein) elicited smaller or no responses in the quadriceps muscle.

The quadriceps changes described can be readily proved to be due to a discharge from the anterior horn cells of the spinal cord:

(1) They are absent in the denervated quadriceps.

(2) They are obtained unchanged when access of the drug to the quadriceps is prevented by cutting off the blood supply to the hindlegs.

(3) As explained, they are not related to the associated circulatory and respiratory changes, but depend on the concentration of acetylcholine reaching the central nervous system.

It may be concluded that the drug acts on elements in the central nervous system, leading to increased motor-cell activity. The response is of the 'd'emblée' type (like the flexor reflex), but the duration of activity in the neurones which are initially excited presumably varies considerably, thus accounting for the gradual relaxation and the different types of relaxation curve.

Action of acetylcholine on the knee jerk

Fig. 2. Cat, 2·6 kg. Decerebrate. Sciatic nerves cut. Records from above downwards: knee jerk elicited from right leg once in 9 sec.; tension of innervated unstimulated left quadriceps; blood pressure; signal; time 10 sec. At signal, 50 μg. of acetylcholine injected into the central end of left subclavian artery.

If acetylcholine is injected intra-arterially while the knee jerk is elicited from one limb, the other showing decerebrate rigidity only, both quadriceps muscles initially react in the same way by the rise of tension already described (Fig. 2). The rise of tension produced by tapping the patellar tendon above the new, raised, level of quadriceps tone is diminished initially and may be abolished. As the quadriceps relaxation phase sets in, the knee jerks become progressively bigger, and may, rarely (as in Fig. 2), become greater than before the injection. These changes are central in origin, and are also obtained after cutting off the blood supply to the hindlimbs. This type of result presumably depends on central occlusion. The motor-neurone pool (supplying the quadriceps) which is stimulated by the injected acetylcholine, initially includes all (or most of) the anterior horn cells stimulated during the knee jerk. At this stage therefore no (or small) summation of the acetylcholine contraction and the knee jerk takes place. As the acetylcholine effect wears off, the occlusion becomes partial; some of the cells of the knee jerk motor-neurone pool remain under sustained acetylcholine stimulation, while others are only activated during the knee jerk itself. The later potentiation may represent, perhaps, a residual acetylcholine 'subliminal' effect.

Effect of atropine on the responses to acetylcholine

Atropine in doses of 1 mg. annuls the central response to acetylcholine; the effect reaches its maximum gradually over a period of 10 min., and then this depression gradually wears off over the next 30–50 min. The results of a typical experiment are illustrated in Fig. 3; (A) shows the type of response obtained prior to atropine. After 5 min., (B), when the depressor action on the circulation was almost completely annulled, repetition of the intra-arterial injection of acetylcholine produced a central effect, which, though little diminished in magnitude, was very transient in character. At 8 min., (C), the response was almost completely abolished, though the general level of blood pressure and the circulatory reactions were similar to those observed at 5 min. At 15 and 25 min., (D) and (E), recovery was progressively taking place. At 50 min., (H), the central motor response initially exceeded the pre-injection level; the duration of the response was, however, still considerably diminished. If the total area of the myogram curve is taken as a measure of the total motor-neurone discharge under the influence of the drug, then even at this late stage some depression by atropine of the central stimulating action of acetylcholine is still evident. Sometimes, 1 hr. after the injection, spontaneous changes in muscle tone set in, consisting of fluctuations rising above the base-line. These effects of atropine are centrally produced, as the doses employed do not modify the response of the denervated muscle to acetylcholine, when appropriate amounts of that drug are given by the method of close intra-arterial injection. Though atropine ultimately annuls the action of acetylcholine, both on the central nervous system and on the circulation, the time course of the effects differs considerably in the two systems.

Action of eserine on quadriceps muscle tone

All the experiments were carried out after the injection of atropine (1 mg.). Positive results on injection of eserine (doses up to 4 mg.) were obtained in only one-third of the cases from the innervated quadriceps. Two types of response were observed:

(*a*) There may be a gradual progressive step-like increase in tension setting in after a latent period of 1–2 min. and reaching its maximum about 4–6 min. later. Relaxation is likewise very slow and may be incomplete after 10 min. (Fig. 4). In these cases, eserine (unlike injected acetylcholine) is gradually

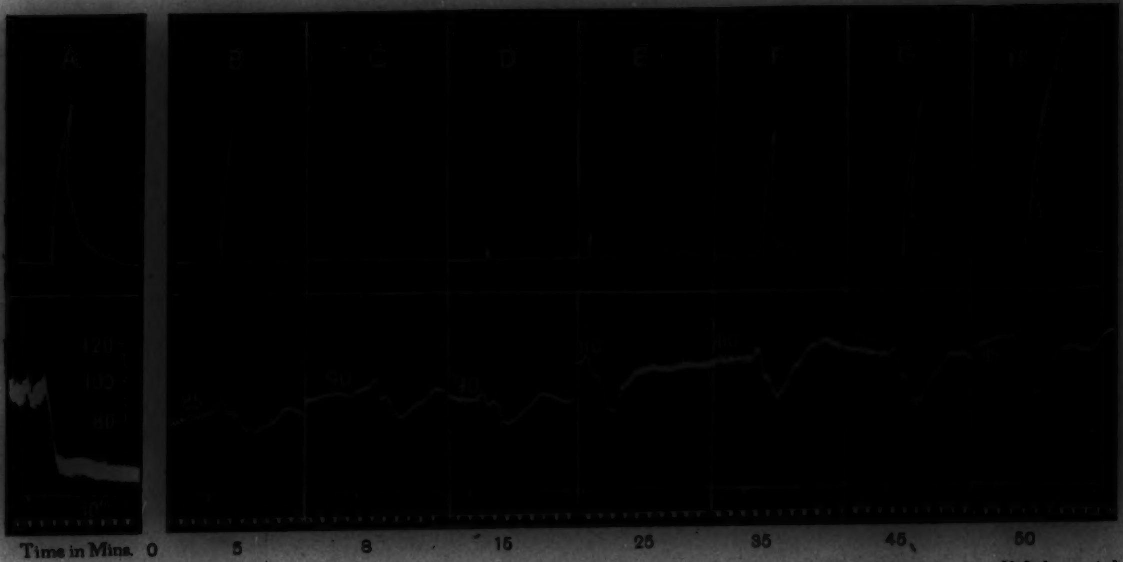

Fig. 3. Cat, 3·2 kg. Decerebrate. Sciatic nerves cut. Records from above downwards: tension of right innervated quadriceps; tension of left denervated quadriceps; blood pressure; signal; time 10 sec. At A, B, C, D, E, F, G, H, 50 μg. of acetylcholine intra-arterially. Between A and B inject 1 mg. of atropine intra-arterially.

'recruiting' motor-neurones into the pool of actively discharging cells; the duration of the discharge likewise varies greatly in different motor-neurones.

(*b*) On the other hand, after a similar latent period, the tension may rise rapidly to its peak, the rise being accompanied by convulsions (Figs. 5, 6). The relaxation phase may then proceed as in (*a*), and the decline of tone may be accompanied by the persistence or the appearance of convulsive movements. The maximal tensions recorded have been about 0·5 kg. above the pre-injection level. The blood pressure usually rises to a moderate extent (e.g. up to 40 mm. Hg) after a very brief latency; sometimes, with the later onset of convulsions, a further rise may take place. Respiration is commonly markedly stimulated. Extension of the forelimbs may occur; micturition or defaecation may be induced.

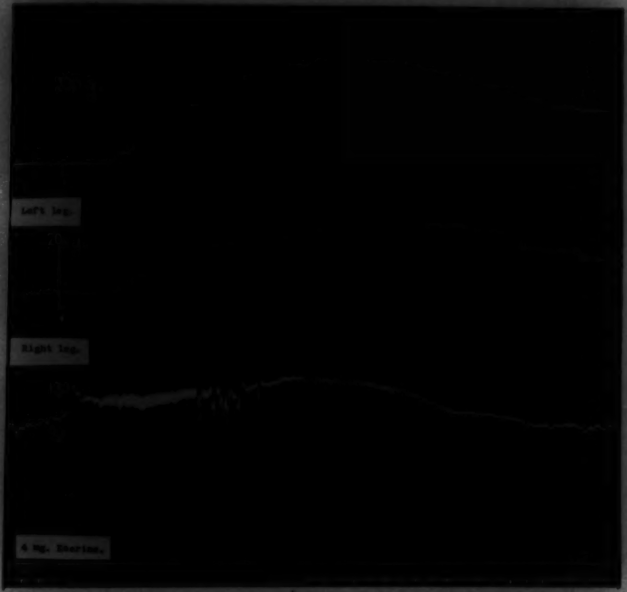

Fig. 4. Cat, 3·5 kg. Decerebrate. Sciatic nerves cut. Atropine 1 mg. Records from above downwards: tension of left innervated quadriceps; tension of right innervated quadriceps; blood pressure; signal; time 30 sec. At signal, inject 4 mg. of eserine intravenously.

On repeating the injection of eserine it is unusual to get a further motor response; positive reactions to a third injection are still rarer.

The changes in muscle tone and the convulsions are due to an action of eserine on the central nervous system. They are unrelated to changes in blood pressure or respiration; they are not observed in the denervated limb; they are observed without essential differences if access of the drug to the limb is prevented by suitable occlusion technique (Fig. 6).

These responses to eserine, it must be remembered, took place in the presence of full doses of atropine.

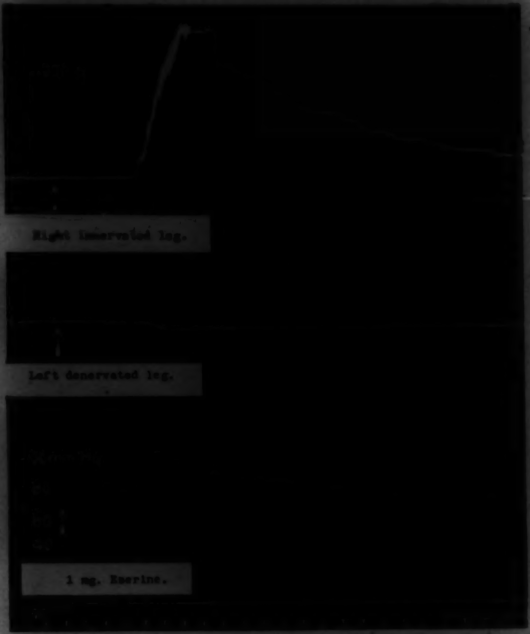

Fig. 5. Cat, 3·0 kg. Decerebrate. Sciatic nerves cut. Atropine 1 mg. Records from above downwards: tension of right innervated quadriceps; tension of left denervated quadriceps; blood pressure; signal; time 30 sec. At signal, inject 1 mg. of eserine intravenously.

DISCUSSION

The results described with acetylcholine on muscle tone are essentially the same as those obtained by Bülbring & Burn [1941] on the isolated perfused spinal cord of the dog. Schweitzer & Wright [1937 *b*, *c*] sometimes noticed an initial stimulation of the knee jerk in the chloralosed cat, but their main finding was inhibition. In this series of experiments we observed no depression of the reflex arc of the knee jerk by acetylcholine.

Atropine annulled the central excitatory effects of acetylcholine, as it had been observed previously to annul the central inhibitory action of the drug.

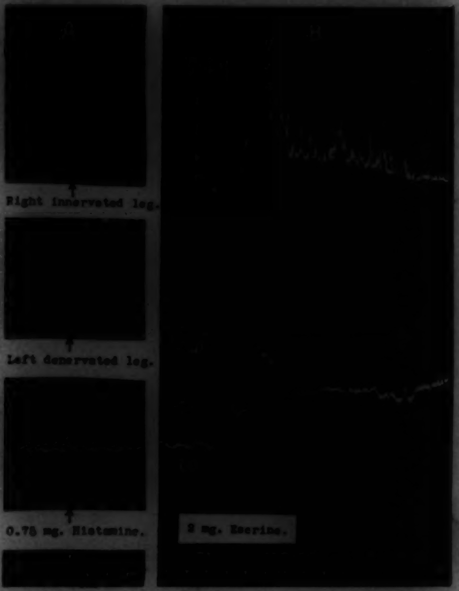

Fig. 6. Cat, 3·0 kg. Decerebrate. Sciatic nerves cut. All branches of the aorta below the renal arteries tied. Clamp on abdominal aorta throughout. Atropine 1 mg. Records from above downwards; tension of right innervated quadriceps; tension of left denervated quadriceps; blood pressure; signal; time 30 sec. At A, 0·75 mg. histamine injected intramuscularly into one leg to test the degree of ischaemia of the limbs. At B, 2 mg. of eserine injected intravenously.

The outstanding feature of the results with eserine was their unpredictability, in the sense that, in many experiments, no changes in muscle tension were produced. In the cat, under chloralose anaesthesia, eserine produces marked and even violent excitatory responses in the great majority of experiments. It seems, therefore, that chloralose in the cat considerably potentiates the central excitant action of eserine. There is evidence that eserine may have a central depressant action; Schweitzer & Wright [1937*b*] found an initial central inhibitory action in a few chloralosed cats; this effect was on occasion marked and prolonged, but was followed by the usual excitation. Bülbring & Burn [1941], working with the isolated perfused spinal cord of the dog and Merlis & Lawson [1939] with chloralosed dogs, found that eserine inhibited the knee jerk. It is conceivable that some balance of central inhibitory and excitatory actions may account for our many negative results with eserine; but we must confess that we never observed a pure inhibitory response in this series. We feel that, at this stage of our knowledge of the pharmacological action of acetylcholine and eserine on the central nervous system, care should be taken to avoid general conclusions based on a single species, or on experiments carried out under one set of conditions. It is probable that the species, the preparation, the anaesthetic employed, the response studied and other undefined factors may play a part in determining the ultimate effect observed.

SUMMARY

1. The action of acetylcholine, atropine and eserine was studied on the decerebrate cat.

2. Intra-arterially injected acetylcholine (25–100 μg.) causes a discharge from the central nervous system, resulting in an increase of tone in the innervated quadriceps. A similar increase in tension was also observed in the quadriceps from which the knee jerk was elicited.

3. Atropine at first abolishes or diminishes the response to acetylcholine for a period of 45–60 min.; later 'spontaneous' changes in tone may occur.

4. Eserine is excitatory to the central nervous system of the atropinized decerebrate cat in a number of cases, although its action is irregular and unpredictable.

5. Attention is drawn to the striking differences in the action of these drugs in different species, in different preparations and under different anaesthetics.

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STIMULUS INTENSITY IN RELATION TO EXCITATION AND PRE- AND POST-EXCITATORY INHIBITION IN ISOLATED ELEMENTS OF MAMMALIAN RETINAE

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Since the demonstration of inhibition in the whole optic nerve by Granit & Therman [1935] and in single fibres of the frog's eye by Hartline [1938], it has been evident that the primary effect of illumination of the light receptors emerges in the optic nerve, after passage through the synaptic layers, in the shape of complicated patterns of excitation and inhibition. Such complications do not occur in the simple non-synaptic retina of the horseshoe crab *Limulus* [Hartline & Graham, 1932] where the discharge—just as in other sense organs—is an even flow of impulses, at higher intensities of stimulation interrupted by a brief silent period.

In the frog's optic nerve inhibition—below to be termed pre-excitatory inhibition—is present in its purest form in about 30% of the fibres which merely respond with an off-effect when the stimulus is withdrawn and which always are completely inhibited by illumination. Pure excitation is present in less than 20% of the fibres; these respond with an even flow of impulses as in *Limulus*; in other words, they are elements which behave as if they merely had transmitted the primary excitation pattern of the initial receptor mechanism. But the majority, more than 50%, are of the combined excitation-inhibition type responding with both on- and off-discharge. The on-off response is therefore the typical and characteristic discharge in the single fibres of the frog's optic nerve. The percentages given are from Hartline's work in which the types described also were found to be stable.

In the course of work on colour reception with mammals [summarized by Granit, 1943*a*], I have had occasion to observe a large number of isolated fibres in the optic nerves of, particularly, cats [Granit, 1943*b*] and guinea-pigs [Granit, 1942], the former a mixed (rod-cone) eye with impressive rod dominance, the latter a pure rod-eye. These observations have later been supplemented with a number of systematic intensity series in the two states of adaptation. These two types of retinae were very different, both when compared with each other and when individually compared with the frog's eye. In particular, the inhibitory phenomena, which in an interesting manner connect this field with general neurophysiology, turned up with new aspects. This work is a presentation of the results.

METHOD

The cats were decerebrated, the guinea-pigs anaesthetized with 20% urethane (4–6 c.c.). In some experiments rats were used [cf. Granit, 1941]. After decerebration the cats were also given a small dose of urethane (about 10 c.c.) to keep eye movements in check. Cornea and lens were removed and a platinum wire micro-electrode, isolated with glass down to the tip, was inserted under a binocular preparation microscope with the aid of a micromanipulator. Leads were taken to amplifier, cathode ray, and loud-speaker in the usual manner.

In the standard experiments the animals were dark-adapted (scotopic animals) and the whole eye stimulated with a lamp at 892 m.c. with an energy distribution corresponding to 2,800° K. Ilford neutral filters and a neutral wedge were used for weakening the strength of this stimulus. Light-adaptation (photopic animals) for 10 min., when needed, was carried out with the lamp mounted in the preparation microscope (2,400 m.c.). This light was also sometimes used as maximal stimulus.

RESULTS

1. *The guinea-pig's retina*

All three mammals studied in the colour work (cat, guinea-pig, rat) share the property of having very few fibres that could be characterized as pure off-elements. Against the 30% of such elements in the frog's eye stand 1 or 2%, as a rough estimate, in the mammals studied. These pure off-fibres generally have had very high thresholds, in the neighbourhood of 1000 m.c.

The pure rod-eye of the guinea-pig differs from the mixed retinae of the cat and the frog in that the great majority of the isolated spikes belong to the pure excitation type, illustrated in Fig. 1. The discharge is an even flow of impulses, as in the eye of *Limulus*, at higher intensities often, though not always, interrupted by a silent period after the initial high-frequency start. These elements are of two types: (i) the upper type, illustrated in *a*, responds precisely as the *Limulus* fibres, with high initial frequency and little or no after-discharge; in the lower type, illustrated in *b* (continued in third record), the discharge ends with an after-discharge which in this case is particularly well developed. The same element responds in *c* after light-adaptation. A typical effect of light-adaptation is the shortening of the after-discharge. The diminution of spike height in the middle of the record is probably caused by a minute eye-muscle contraction. The after-discharge in record *b* should be clearly distinguished from an off-effect (see Fig. 8). The latter represents a real increase in frequency of the discharge at 'off' and is wholly or temporarily inhibited by re-illumination. The after-discharge, however, is a gradual disappearance of the effect of the stimulus. It is uninfluenced by re-illumination unless the pause of darkness is long enough for recovery of the element and fresh on-discharge.

These pure on-elements, which respond with a continued discharge as soon as stimulus intensity is sufficiently high, form about 90% of the elements in the guinea-pig's eye. The rest of the elements are practically all of them on-off elements such as those in the cat's eye, but responding within a considerably smaller frequency range.

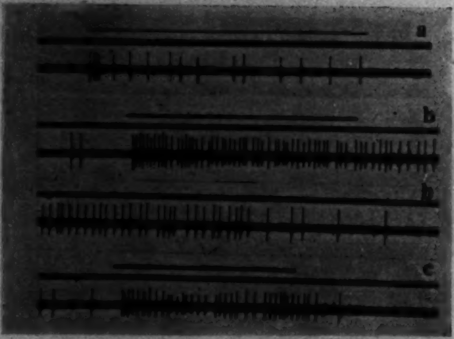

Fig. 1. Micro-electrode record from the optic nerve of the guinea-pig serving to illustrate pure on-elements giving continuous discharge. Light signal and time in 1/50th sec. above each record in this and all figures to follow: *a*, scotopic retina and element responding to 12·3 m.c.; *b* and *c*, from another experiment, *b* scotopic, *c* photopic, in both cases responding to 18·2 m.c. See text.

The characteristic relations between stimulus intensity and impulse frequency are illustrated in Fig. 2 for four such on-elements in the dark-adapted state (filled circles), the lowest, D, over a range of 6 logarithmic units. The variations in the absolute threshold probably depend upon the degree of anaesthesia, which is difficult to control as animals react very differently. The uniform results are: that the variations in impulse frequency take place over a range of about 1–100 impulses per sec., and that the curves very soon cease to rise with increasing intensity, some even bending down at intensities of a relatively moderate order of magnitude (1000 m.c.).

The elements B, C and D were light-adapted and the same experiment repeated (empty circles). Light-adaptation pushes up the threshold so that the frequency range of up to 100 impulses per sec. is compressed within a smaller intensity range. In B light-adaptation was continuous and merely interrupted for a few seconds during which the test stimulus at different intensities was introduced. In the curve marked 'circle with cross' each observation followed 1 sec., in the other one (empty circles) 2 sec. after cessation of the light-adapting stimulus.

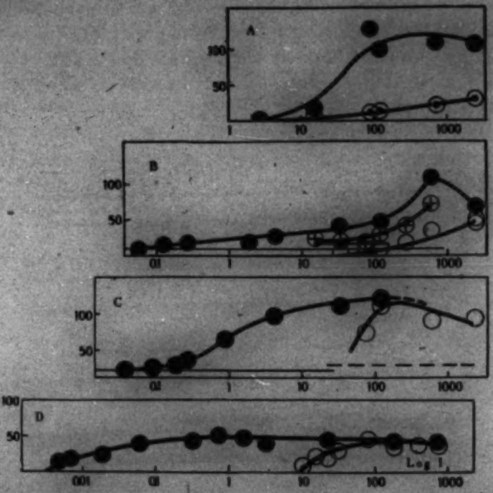

Fig. 2. Guinea-pig. Micro-electrode. Frequency of the discharge of isolated elements plotted in impulses/sec. against log intensity in meter candles. A, scotopic; upper curve initial frequency, lower curve frequency counted after silent period or corresponding interval. B, filled circles scotopic, open circles photopic (see text). In this and the following curves ordinates =initial freq. Frequency of spontaneous activity, when present, indicated by horizontal lines, separately for scotopic and photopic state. C and D, separate experiments marked as in B.

The effects of light-adaptation may now be briefly summarized: (i) after-discharge is reduced, (ii) threshold increased, (iii) on an average, the frequency-log intensity curves would probably also rise at a slightly steeper gradient than in dark-adaptation.

2. *The cat's retina*

General observations. The cat's retina proved to be a far more complex organ than that of the guinea-pig. Some 20% of the elements are of the pure excitation on-type, described above for guinea-pigs. But their upper frequency limit is higher than in the guinea-pig. The rest of them are of the on-off type. These behave in a complex manner when stimulus intensity is varied.

In both cats and guinea-pigs some spontaneous activity is common in the spike located by the electrode. They differ in this respect from the frog, in which spontaneous activity is less marked, though it often turns up after some dark-adaptation. This slow spontaneous activity can be made very useful for the experimenter: inhibitory phenomena, which otherwise would be unsuspected, are recognized by the decreased or fully inhibited spontaneous rhythm. In order to illustrate this, Fig. 3 should be consulted. The intensity is increasing downwards and the numerals on the right indicate the densities of the filter-wedge combination in front of the 892 m.c. stimulus. In the two lowest pictures (1·38 and 0·09) the spontaneous frequency has decreased somewhat, due to a protracted inhibitory after-effect of the strong stimuli used. The rate of discharge in the on-effect increases continually downwards and reaches frequency values around 300 per sec. The frequencies but rarely surpass this figure though individual receptors have reached 400. The off-effect behaves in a very complicated fashion. The frequency first increases slightly above the spontaneous rhythm, then diminishes, rises a second time (at density 1·38) and

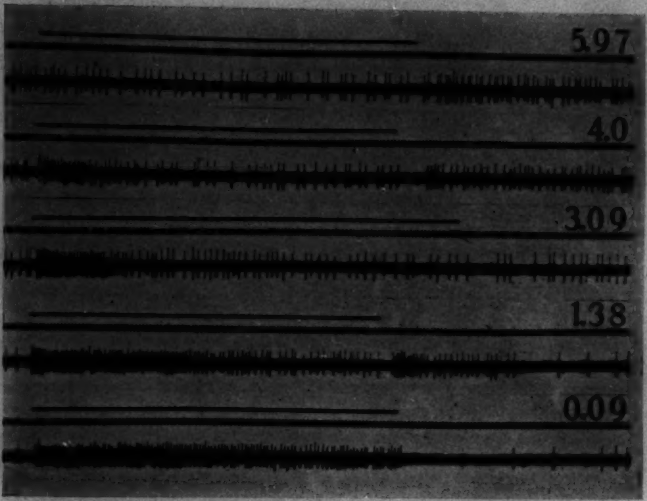

Fig. 3. Cat. Isolated spontaneously active element responding to downwards increasing intensities. The numerals to the right show the extinction (densities) of the filter-wedge combination used in front of a light of 892 m.c. Owing, partly, to some light-adaptation caused by stronger stimuli the spontaneous rhythm decreases downwards.

is finally completely blocked at the highest intensity. The pause in the spontaneous rhythm shows that it then is actively inhibited.

Another element is shown in Fig. 4. In this case there is very little spontaneous activity. The on-effect increases in frequency downwards, reaches a maximum, decreases (at density 3·01) and in the final lowermost record at the highest intensity, the whole on-discharge is very much delayed, as if somehow it had managed to escape an initial inhibition. The off-effect increases in frequency until a maximum is reached at density 3·01 and then slightly decreases at the maximal intensity. This type of element is very common in the cat's eye, probably the most common type. In the background of the same record is seen a brief initial discharge of short latency, caused by other elements less well localized by the micro-electrode but sufficiently many to give a small group of spikes of brief latency. This is often noticed.

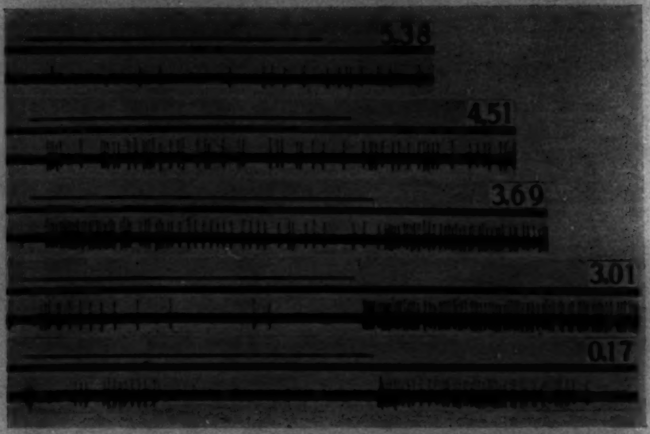

Fig. 4. Cat. As in Fig. 3, but another experiment with another type of on-off element.

Intensity-frequency curves for the two states of adaptation. Some experiments on the effect of stimulus strength upon the frequency and the total number of impulses at 'on' and 'off' have been quantitatively analysed in Figs. 5 and 6 for the two states of adaptation. Light-adaptation of the animal (wide open pupil) has been achieved with the aid of the lamp (2,400 m.c.), used for insertion of the micro-electrode. There is a full description of the experiments in the figure texts. In Fig. 5 B the two lines parallel with the abscissa represent the frequency level of the spontaneous activity of the spike studied. The broken horizontal line refers to light-adaptation, the line drawn in full to dark-adaptation. Thus when the curves go below these lines there is active inhibition of the spontaneous rhythm. The curves should be compared with the much simpler curves of Fig. 2 for the guinea-pig. Clearly rivalry between excitation and inhibition plays a much greater role in this mixed retina than in the guinea-pig's pure rod retina.

Pre- and post-excitatory inhibition. These two terms are easily understood. By *post-excitatory inhibition* is meant the kind of inhibition that follows *after* excitation (by light) and which leads to suppression of spontaneous activity and off-effect, if an off-effect has been present at some lower intensity level. This kind of inhibition is common in the retinae of *both* cats and guinea-pigs, perhaps more common in the latter. It is illustrated in Fig. 3, lowermost record, and in Fig. 5 B it is seen to press the intensity-frequency curve for the off-effect in the dark-adapted state below the level of spontaneous activity (in the region of about 1 m.c.). Post-excitatory inhibition thus cuts down after-effects of excitation which otherwise may have occurred. In Fig. 7 post-excitatory inhibition is illustrated for a pure on-element with some spontaneous activity (guinea-pig).

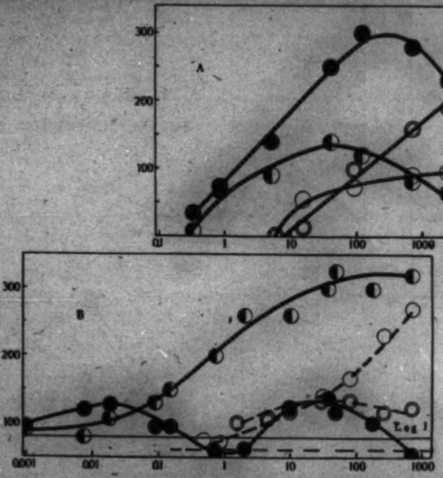

Fig. 5. Cat. Frequency of discharge of isolated elements plotted in impulses per second (initial freq.) against log intensity (m.c.). Light- and dark-adaptation compared for two elements (A and B). Scotopic on-discharges = semi-filled circles; scotopic off-discharges = filled circles; photopic on-discharges = open circles; photopic off-discharges = circles with double contour. Spontaneous frequency present for element B and marked by horizontal line in full (scotopic) or interrupted (photopic).

Post-excitatory inhibition explains a number of earlier observations by other authors: with the eye of the horned toad Meservey & Chaffee [1927] noted that the fast positive off-effect of the electroretinogram diminished at higher intensities, as also found by Wrede [1937] and Therman [1938] with the frog's electroretinogram. Hartline [1938] showed that the off-effect in single fibres of the optic nerve of the frog also diminished at higher intensities, as would be expected from the general correspondence between the fast positive phases (due to the retinal component potential P II) of the electroretinogram and the discharge in the nerve. The difference in Fig. 1 between the two types of pure on-elements in the retina of the guinea-pig is probably also due to a different

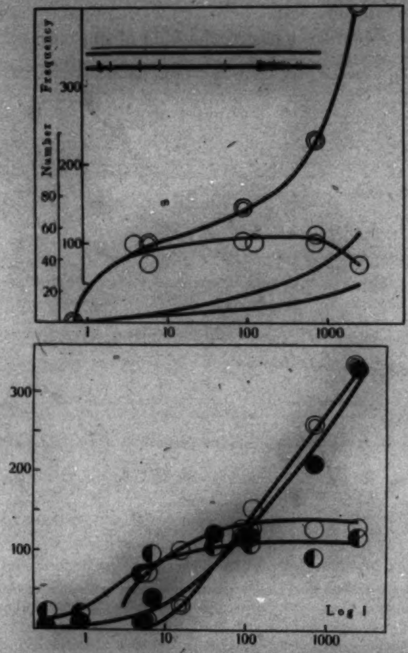

Fig. 6. Cat. Same as in Fig. 5 for two elements, but in addition has been measured, in upper picture, the total number of impulses of the photopic element reproduced in the inset. The discharge in this case was relatively brief at 'on' and 'off'. The lower pair of curves in this picture, for which the individual readings have been left out, show total number of impulses in the records that were used for frequency measurements in the upper pair. Upper curve, off-discharge; lower curve, on-discharge.

degree of development of post-excitatory inhibition in them. The one type (*a*) had no after-discharge whatever, whereas the other type (*b*) still discharged some time after cessation of illumination. Lack of after-discharge may be due to prominent post-excitatory inhibition.

Post-excitatory inhibition may be identical with the 'local stimulatory inactivation', noted after electrical stimulation of the cerebral cortex by Dusser de Barenne & McCulloch [1937] and by them termed *extinction*. It is most conveniently observed in the 'simple' discharge of the guinea-pig's retina when a spontaneously active pure on-element has been localized with the aid of a micro-electrode (as in Fig. 7). These fibres generally stop their spontaneous activity for a while after illumination of the retina with a sufficiently strong light.

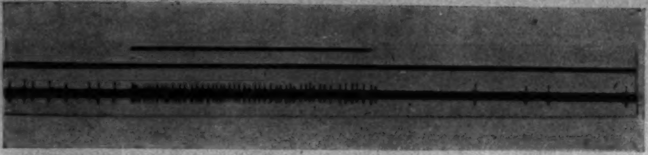

Fig. 7. Guinea-pig. Spontaneously active element giving a pure on-discharge to illumination with 892 m.c. The discharge is followed by post-excitatory inhibition silencing spontaneous rhythm for a while.

Pre-excitatory inhibition is the type of inhibition originally described by Granit & Therman in this *Journal* [1935]. It has the interesting property of reflecting the behaviour of the slow retinal component potential P III [cf. Granit, 1938]. In order to differentiate it from post-excitatory inhibition it is termed *pre*-excitatory, the term emphasizing its brief latent period, briefer than the latent period for excitation, as is best demonstrated by re-illumination on top of an off-effect. In work, now in progress, the two forms of inhibition have been found to possess very different properties with respect to diverse agents.

In order to be demonstrable pre-excitatory inhibition needs the background of excitation which, for post-excitatory inhibition, was provided by spontaneous activity. But for pre-excitatory inhibition the best background is the off-discharge following cessation of illumination, and the test for the efficiency of the inhibition therefore consists in re-illumination after a brief pause of darkness. The pure off-element that is inhibited by light and set free by cessation of illumination exhibits pre-excitatory inhibition in pure form. In this element it is so effective that every sign of a discharge to illumination, started by the primary sense-cell, is curtailed in the synapses before it has had a chance of turning up in the optic nerve. The off-effect is therefore also immediately and completely inhibited by re-illumination.

Two elements illustrating typical aspects of pre-excitatory inhibition in cats are shown in Fig. 8. There is a slight spontaneous discharge in *a* which is increased into an on-discharge upon illumination and again accelerated into a vigorous off-effect upon cessation of illumination. The on-effect is insignificant compared with the off-effect. The stimulus is here 892 m.c. diminished by *red* filter and neutral filter (density 1·25). In *b*, when illumination is repeated after removal of the neutral filter, pre-excitatory inhibition is relatively stronger than excitation so that at 'on' there is merely inhibition, as demonstrated by the decreased spontaneous activity. A part of the film has been cut out and then follows the off-discharge. Re-illumination after a brief pause completely inhibits this off-discharge, shown again as control at the end of record *b*. The red filter is finally removed in *c* so that light from the whole spectrum stimulates the eye. There is now in response to illumination a very much delayed discharge, looking as if it barely had succeeded in escaping from pre-excitatory inhibition. The final record *d* is another element from another experiment. It shows re-illumination during the off-discharge of an on-off element. Here the pre-excitatory inhibition, elicited by re-illumination, merely succeeds in causing a brief pause of inhibition in the off-discharge. This pause is interrupted by a fresh on-discharge of this on-off element.

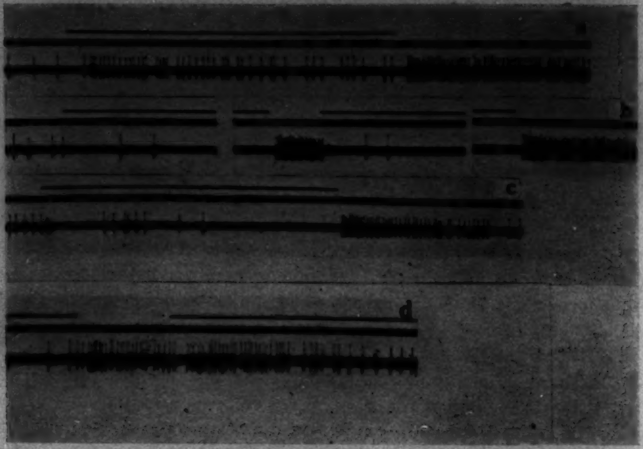

Fig. 8. Cat. Analysis of pre-excitatory inhibition by re-illumination during off-discharge of on-off element. Fully described in the text. The records *a*, *b* and *c* belong to the same element, record *d* to another.

What would now in cats be the *typical* behaviour of pre-excitatory inhibition in the test by re-illumination? Would it be a *complete inhibition* as in *b* or merely a *brief inhibitory pause* as in *d*? Actually the large majority of elements are of the latter type, giving merely a brief inhibitory pause. Pre-excitatory inhibition in the cat's retina is better developed in the upper intensity ranges (cf. Figs. 5, 6).

Pre-excitatory inhibition presupposes on-off elements and is maximally developed in the pure off-elements. For this reason it is of far less significance in the rod retina of the guinea-pig which chiefly contains on-elements. Again, in the retina of the frog (rods to cones about 50 : 50), pre-excitatory inhibition is far more prominent than in the retina of the cat where the cones are few, though sufficiently many to give a Purkinje shift [Granit, 1943*b*].

The degree of development of the off-discharge in a given retina is a rough index of the use it makes of pre-excitatory inhibition. The reason for this statement is that the off-discharges can be regarded as a consequence of pre-excitatory inhibition. The pure off-elements are inhibited by light and are piling up inhibition during illumination so that the longer the duration of the exposure the greater, within limits, the frequency and duration of the off-effect. In the cat's eye pure off-elements are rare and consequently complete inhibition of the off-effect by re-illumination is far less common than the brief inhibitory pause, illustrated in Fig. 8*d*.

Stable and labile units. In the frog's eye Hartline [1938] found on-fibres, off-fibres, and on-off fibres to preserve their type despite variations in intensity of the stimulus. The suppression in this eye of the off-discharge by high intensities does not mean that the isolated unit has changed type but is probably due to post-excitatory inhibition, as suggested above. In the retinae of cats and rats the pure on-elements have also been found to be stable. However, the on-off elements in the cat's retina are variable. At high intensities they often behave as pure off-elements, sometimes also as pure on-elements (as in Fig. 3); at low intensities the on-effect may predominate, and as the frequency-intensity curves show, rivalry between excitation and inhibition may at different intensity levels give the discharge a very different aspect. The few pure off-elements in the mammalian eyes are also stable but, since their thresholds as a rule are very high, they can only be studied within a very limited range of intensities.

In the cat's eye the on-off elements behave as if they were a combination of pure on-elements with pure off-elements. At the moment it is impossible to explain the variability of these elements. But the facts (i) that the negative component P III of the electroretinogram is a high-intensity component in cats and (ii) that this component generally is associated with inhibition suggest that the great increase of pre-excitatory inhibition at high intensities may account for at least some of the complex changes in the on-off elements at such intensities.

DISCUSSION

Comparison with electroretinogram

The off-effect in the slow retinal potential (the electroretinogram) is well developed in the frog, which has a large number of pure off-elements represented in its optic nerve; it is much smaller in the cat's electroretinogram. The first effect of re-illumination on top of a retinal off-effect is a large negative *a*-wave (component P III) in the electroretinogram of the frog, but merely a slightly delayed, sub-normal positive *b*-wave (component P II) in the electroretinogram of the cat. In the frog this negative *a*-wave of P III is coincident with pre-excitatory inhibition in the nerve. This inhibition, according to Hartline [1938], is complete for the pure off-elements of this animal. However, in the cat, which has chiefly on-off elements, the inhibition, caused by re-illumination, is generally a brief pause in the off-discharge (Fig. 8*d*) followed by the discharge of the reactivated 'on'-fraction of the complex on-off element. Complete inhibition is rare in this animal. These comparisons suggest that the negative *a*-wave of the electroretinogram (component P III) in re-illumination is well marked merely when—as in the frog—pre-excitatory inhibition in the individual elements is so well developed that a large fraction of the off-discharge of the whole retina is completely inhibited by light. Similarly the off-effect of the electroretinogram also is well developed in retinae in which a large number of individual elements actually respond merely at 'off'. It was shown by Granit & Therman [1937] that in this case there is also in the off-effect of the electroretinogram re-activation of the positive component P II. This fact has been incorporated in the recent analysis of the electroretinogram of the frog's eye, published by Bernhard [1942].

In all comparisons between the electroretinogram and the discharge of isolated elements it should be remembered that the former is an average effect and that it is necessary in such comparisons to be able to distinguish the common from the atypical in the properties of isolated units. Records from the whole nerve supplement the information from single fibres. Recently Adrian [1941] has taken such records from the whole optic nerve of cats and points out that bright light tends to give a brief on-discharge followed by inhibition, as in Fig. 4 (lowermost record). This is in good agreement with my experience that pre-excitatory inhibition at high intensities characterizes most isolated units in the optic nerve of the cat. Furthermore, it agrees with the fact that the component P III (in the electroretinogram), which is associated with pre-excitatory inhibition, is a high-intensity component in cats [Granit, 1933] and that the cat's electroretinogram at high intensities often has a diminished *b*-wave of P II, followed by a negative phase of P III.

Rods and cones, duplicity theory

The duplicity theory comes into the foreground because of the differences between cats and guinea-pigs. Since these differences are post-synaptic, interpretation of the results in the light of the theory presupposes the assumption that the synaptic organization is different for rods and cones, an assumption well justified by retinal histology. The main conclusion that would be suggested by these experiments is that from the functional point of view the synaptic organization of the rods is simpler and for this reason more likely to transmit the receptor effect without other modifications than those caused by summation, due to convergence, and by post-excitatory inhibition—which, after all, may be paralleled with similar phenomena in peripheral nerve. Hence would arise the absolute dominance of the simple on-elements in the rod-retina of the guinea-pig. The presence of a few off- and on-off elements in the guinea-pig's rod-retina would then serve to emphasize that these as well as all other experiments with the micro-electrodes, those on colour reception [Granit, 1942–3] as well as those on adaptation (unpublished), suggest that there are transitional forms between rods and cones (in the strictest sense of the terms). The guinea-pig has no Purkinje shift and few if any so-called red modulators, elements sensitive to red within a relatively narrow spectral region, and thus can hardly be held to have any cones.

The cat has cones, though these still are in the minority but, functionally, they are easily detected with the micro-electrode technique. A certain number of the elements of the cat's retina show a definite Purkinje shift and, after light-adaptation, respond to spectral light with the sensitivity distribution of the so-called photopic dominator of the cones with maximum at 0·560μ [Granit, 1943*b*]. Pure cone-eyes have only been studied in cold-blooded animals [Meservey & Chaffee, 1927; Bernhard, 1941] but these have all had prominent off-effects and prominent negative components in their electroretinograms, as the many correlations between this component (P III) and pre-excitatory inhibition would have led one to believe. Thus there are reasons for assuming pre-excitatory inhibition to be a feature of the synaptic organization of the cones. Nevertheless we must ask whether the differences between cats and guinea-pigs could be due to the heavier anaesthesia of the latter.

The rat, anaesthetized with urethane, as the guinea-pig, has a somewhat greater percentage of on-off elements than the latter and for this reason was chosen for some experiments on the effect of this drug. Three urethanized rats, in which first a place giving a well marked on-off discharge of a number of elements had been localized with the micro-electrode, received three times the normal dose in two intraperitoneal injections at an interval of 10 min. The elements under the electrode continued to react as on-off elements despite the abnormally deep anaesthesia. A similar experiment with the same outcome was performed with a cat which was given successive doses of urethane until its respiration stopped. These experiments showed that very much larger doses of urethane than those used for guinea-pigs did not succeed in removing off-discharges. It is therefore improbable that the small number of on-off elements in the guinea-pig could be due to selective removal of off-discharges by this drug, even if a small effect of this type were present and served to emphasize the difference between cats and guinea-pigs with respect to pre-excitatory inhibition.

The striking differences in the intensity-frequency curves between cats and guinea-pigs are quite in harmony with what we should expect on the basis of the assumption that frequency is the main determinant for visual effects depending upon intensity. All visual work, on brightness discrimination, flicker fusion, visual acuity, etc., also shows that these functions plotted against log intensity are characterized by much steeper slopes in the cone-region than in the rod-region of stimulus intensities, as would be a direct consequence of the differences in the frequency-intensity curves for cats and guinea-pigs. Still, in this case, it is very probable that the curves actually have been somewhat distorted by the anaesthetic given to the guinea-pigs. The urethanized guinea-pig may not have been able to respond with as high frequencies in the upper ranges as the decerebrated cats could do.

From the point of view of visual discrimination it is clear that a retina possessing the variable receptive pattern of the cat's eye must respond to every fluctuation in intensity with a transformation of the pattern delivered to the cortex. Hardly two elements in this eye are exactly alike! The question as to how retinal interaction can co-exist with a high degree of discrimination, first raised by Adrian & Matthews [1928] and Granit & Harper [1930] and still occupying Bartley [1941], can now be answered. The retinal discharge from the smallest area is differentiated to a degree that was not understood ten years ago. Hartline's work on frogs [1938, 1940*a*, *b*] and these results with the cat's retina place impulse frequency and transformation of pattern into the foreground in every explanation of discrimination.

In connecting the differences between the individual elements of cats and guinea-pigs with an extended duplicity theory incorporating inhibition, I do not wish to convey the impression that this interpretation, based on so few types of retinae, needs be final. I would rather like to express the hope that sooner or later similar experiments will be carried out in other laboratories with other retinae and the same technique. The duplicity theory is, after all, the first purely visual theory that has to be put to a test with the new technique, the more so as this first attempt to do it has shown that striking differences between different retinae can be demonstrated.

SUMMARY

1. In the pure rod-retina of the guinea-pigs an overwhelming majority of the elements merely discharge to onset of illumination. Some of them stop their discharge abruptly upon cessation of illumination, others possess a definite after-discharge gradually diminishing in frequency.

2. In the mixed retina of the cat the great majority of the elements respond as on-off elements, i.e. to both onset and cessation of illumination. Some 20% of the elements behave as pure on-elements. Pure off-elements, merely responding to cessation of illumination, are rare in the eyes of both cats and guinea-pigs.

3. The relative preponderance of on- and off-discharges in the isolated on-off elements of the cat's eye varies a great deal with stimulus intensity.

4. Light-adaptation influences the discharges of the different elements in the following manner: (i) the threshold increases, (ii) an after-discharge, when present, shortens in duration, (iii) similarly the discharges at onset and cessation of illumination are shortened in duration, (iv) the same maximal frequencies are reached in both states of adaptation but the increased threshold in the light-adapted state compresses the range of intensities within which the frequency of the discharge can vary. Hence the frequency-intensity curves will, on an average, rise at a steeper rate in the light-adapted state.

5. In both animals spontaneous activity of the isolated element is common, and suppression of this activity is a good index of inhibitory phenomena which otherwise would escape notice.

6. *Post-excitatory inhibition* is a kind of extended suppression of all activity following *after* the activation of a single element, especially well marked if the stimulus has been strong. It is found in both eyes and in all types of elements.

7. *Pre-excitatory inhibition* has a briefer latent period than excitation and is responsible for the inhibition that in its purest form is found in the pure off-elements which are silenced by illumination and released into activity upon cessation of illumination. Off-effects are thus a sign that pre-excitatory inhibition is present in a given case. Pre-excitatory inhibition leads to suppression of the off-effect by re-illumination. In some on-off elements (cats) this suppression is complete, in most elements, however, merely a temporary block preceding re-excitation.

8. Pre-excitatory inhibition coincides with an active negative component P III of the electroretinogram.

9. The differences in the distribution of excitation and pre- and post-excitatory inhibition over the different elements in the retinae of cats and guinea-pigs are discussed in the light of an extended duplicity theory.

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THE ROLE OF THE PERIPHERAL STUMP IN THE CONTROL OF FIBRE DIAMETER IN REGENERATING NERVES

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Introduction

The process of regeneration of a nerve will only be complete if it leads to the production of fibres comparable in number and size to those normally present in the nerve. Gutmann & Sanders [1943] have found that the characteristic group of fibres of large diameter in the peroneal nerve of the rabbit reappears in the peripheral stump about 200 days after the nerve has been interrupted by a simple crush with forceps. After severance and suture of the nerve, however, the characteristic bimodal distribution of fibre sizes does not reappear, even after one year of regeneration, although some of the fibres reach their full normal diameter.

Holmes & Young [1942] have emphasized that after degeneration each nerve fibre is replaced by a Schwann tube which has about half the diameter of the original fibre. The results of Gutmann & Sanders would be explained if it were assumed that the size of the tube in the peripheral stump had an effect, but not a wholly decisive one, on the final diameter reached by a fibre regenerating within it. The other significant factor would be the amount of axoplasm provided by the central stump; large fibres providing large streams, unless they split up into many small fibres. Thus if large fibres are only produced when large central streams enter large peripheral tubes, we could understand why the normal pattern of fibre sizes is regenerated only after crushing the nerve, for then many or all of the central fibres are left connected with their appropriate peripheral tubes.

But there is no direct evidence available to show that the diameter of the peripheral tubes does in fact exert such a restrictive influence. The problem of the relative importance of the two stumps in deciding fibre diameter can be attacked by making cross-unions of nerves with different fibre sizes, and a series of such experiments is now being undertaken [Simpson & Young].

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Preliminary results [see Young, 1942] show that when a somatic nerve (large fibres) is sutured into the post-ganglionic trunk of the anterior mesenteric nerve (small fibres), myelinated fibres are produced, but they are abnormally small. In the experiments to be described in the present paper, decisive evidence for the influence of the peripheral stump has been obtained in a different way.

The peroneal and tibial nerves of the rabbit each divide low down in the thigh into a number of branches. The peroneal divides into branches supplying the extensor muscles of the anterior aspect of the shank, and the anterior tibial nerve; the tibial into branches supplying the gastrocnemius, soleus, plantaris and other muscles, and the posterior tibial nerve. The anterior and posterior tibial nerves consist almost entirely of sensory and sympathetic fibres. The only muscles supplied by the posterior tibial nerve are the lumbricales and the interossei, all of which are very small, while the anterior tibial nerve of the rabbit is a pure skin nerve. We have thus the situation that both the peroneal and tibial nerves are mixed in the thigh, but divide below into branches which are predominantly either motor or cutaneous. Nageotte & Guyon [1918] and Wohlfart [1938] have shown that these cutaneous and motor branches of the sciatic nerve are distinguishable by the sizes of their constituent fibres, the motor branches containing larger fibres than the cutaneous divisions, observations which have been confirmed by the measurements made during the present work.

This anatomical situation provides an opportunity for studying the effect of the size of the Schwann tubes of degeneration on the size of fibres regenerated in peripheral nerves. When the tibial or peroneal nerve is cut about halfway down the thigh and the stumps rejoined, we have the situation of a mixed nerve being sutured just before two portions containing fibres of specific and differing sizes separate. If the peripheral stump has an influence on the size of fibre regenerated, we should expect to find larger fibres regenerated in the motor branches than in the cutaneous. If, however, the central factor alone decides fibre size, the mixing of fibres in the scar of union will ensure that the two parts of the peripheral stump regenerate fibres of similar sizes. Moreover, we shall be able to evaluate the relative importance of the central and peripheral factors in deciding fibre diameter, by comparing the conditions after suture and after crushing, since in the latter case mixing of the fibres in the scar is at a minimum [see Gutmann & Sanders, 1943].

The observations described in this paper have therefore been made to test whether the peripheral stumps of motor and cutaneous nerves have the power of influencing the diameters of the fibres which are regenerated. We have tested this power of the peripheral stump not only after immediate suture, but also when the stump has been left uninnervated for as long as 15 months. Finally, we have also measured the sizes of the Schwann tubes found in peripheral stumps which have been allowed to degenerate for different periods, in order to discover whether they preserve their relative sizes during the shrinkage which occurs.

METHODS

The experimental animals used were rabbits. All the animals were adults, but varied considerably in size, body weight, and race. A considerable variability in fibre number was found in the nerves examined, but in every case where an abnormally large number was found above the lesion, the specimen had been taken from a large animal, and vice versa. In one animal specimens of the normal peroneal and tibial nerves were removed. In another the peroneal nerves were cut in the thigh on both sides, and the stumps joined again with the concentrated plasma introduced by Young & Medawar [1940]. This animal was then left for a year to recover before it was killed and specimens removed. In four further animals the peroneal nerves were crushed with smooth-tipped forceps at the initial operation, after which they were left to recover for periods of 130–300 days before specimens of the nerve were taken.

In one animal the experiment was designed to allow study of recovery after a long period of degeneration followed by a long period of reinnervation. The tibial nerve was therefore severed in the lower part of the thigh on one side, and the central stump resected for a long distance, avoiding all possibility of reinnervation [Holmes & Young, 1942]. The animal was left for 15 months, and then at a second operation the degenerated tibial nerve was reinnervated by joining with plasma to the central end of the freshly cut and nearby peroneal nerve. As a control, the tibial and peroneal nerves of the other side were cut, and the central end of the peroneal joined to the peripheral end of the tibial. Rejunction of the tibial nerve with its own central stump was prevented by wide resection of the latter. After suture the animal was left for 10 months to recover, and then brought to autopsy.

Finally, two animals were used to compare the sizes of the Schwann tube left in the nerve after short and long periods of denervation. At an initial operation the tibial nerve was cut in the thigh and allowed to degenerate, in one case for 25 days, in the other for 18 months, reinnervation being prevented by resection of the central stumps.

Specimens of the normal or reinnervated nerves were removed from the anaesthetized animals, fixed extended on cards in Flemming's osmo-chrome-acetic mixture, embedded in wax, sectioned transversely at 4μ, and stained with haematoxylin as described by Gutmann & Sanders [1943]. The two specimens of degenerated nerve were similarly fixed and sectioned, but stained to show the endoneurial connective tissue by Heidenhain's picro-blue-black technique, which is a good method for showing Schwann tubes.

Counts and measurements of the *outside* diameters of medullated nerve fibres, and of the *inside* diameters of the Schwann tubes in the degenerated nerves, were made by the sampling technique described by Gutmann & Sanders [1943], except that the measurements were made on photographs of the sections taken at a magnification of 740 ×, instead of on an image obtained by means of a projection microscope.

As in the experiments of Gutmann & Sanders [1943], the following factors were calculated from the counts and measurements of each section: (1) the total number of myelinated fibres (or Schwann tubes) in the section; (2) the number and percentage of fibres greater than 8 μ; (3) the *average-fibre diameter*, obtained by multiplying the number of fibres in each size-group of the sample, by the diameter of the mean fibre of the group, summing the values for the individual size groups, and dividing the result by the total number of fibres in the sample.

The results of these measurements and calculations were recorded in the form of (*a*) tables, and (*b*) frequency block diagrams, in which the number of fibres in each size group was plotted against fibre diameter. The sources of error involved have been discussed by Gutmann & Sanders [1943]. In the present experiments, however, the magnitude of the error of measurement was probably not as great as in the previous observations, especially with the smaller size groups, on account of the larger magnification used.

RESULTS

The fibre composition of the branches of the normal tibial and peroneal nerves.

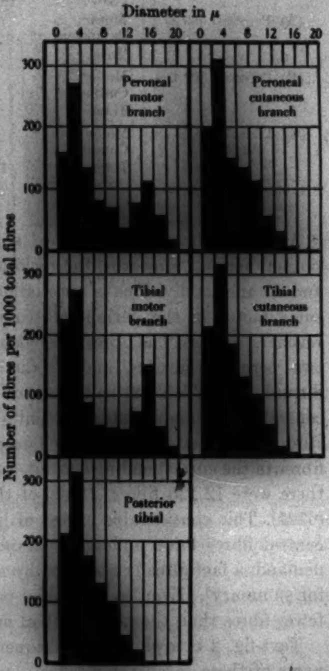
Examination of sections of the normal peroneal and tibial nerves from the lower part of the thigh shows at once that the motor funiculi contain larger fibres than the funiculi which give rise to the anterior and posterior tibial nerves (Pl. 1, figs. 1, 2). Table 1 shows the results of measurements made upon the peroneal and tibial nerves at this level, and upon the posterior tibial nerve in the shank. The motor branches of the peroneal nerve contained 1887 fibres larger than 8 μ (35·6 %), compared with 632 fibres (20·2 %) exceeding this diameter in the cutaneous branches. Similarly, in the motor funiculi of the tibial nerve there were 36·8 % of these large fibres, while the cutaneous funiculi contained only 16·4 %, and the posterior tibial nerve 16 %. The average-fibre diameters were also larger in the motor branches.

Text-fig. 1 is a chart of the relative number of fibres per 1000 total fibres in the various size groups of the two divisions of the peroneal and tibial nerves, and in the posterior tibial nerve. In all parts of these nerves a considerable range of fibre sizes was present, the smallest fibres being 1–2 μ in diameter [see Duncan, 1934]. All the fibres in the group marked 0–2 μ in the histograms lie near the upper limit of the group; hence the 0–2 μ group is really a 1–2 μ fibre group. The upper limit of fibre size was not, however, the same in all

TABLE 1. Numbers and sizes of fibres in the various divisions of the normal tibial and tibial peroneal nerves. Rabbit 928

	Nerve	Total no. of fibres	No. of fibres larger than 8 μ	% large fibres	Average-fibre diameter in μ
Peroneal in thigh	Motor	5,301	1887	35·6	6·97
	Cutaneous	3,128	632	20·2	4·37
Tibial in thigh	Motor	7,263	2673	36·8	6·89
	Cutaneous	8,634	1416	16·4	4·60
	Total	15,897			
Posterior tibial		7,753	1240	16·0	4·59

parts of these nerves. The largest fibres in the cutaneous branches of the peroneal and tibial, and in the posterior tibial nerve, were between 14 and 16 μ in diameter, while the motor branches of both nerves contained fibres up to 20 μ in diameter. Moreover, the actual form of the distribution was different in the two cases. In the cutaneous branches, and in the posterior tibial nerve, the histograms showed a peak in the 3 μ group, and thereafter the number of fibres in each successive group steadily declined, reaching zero at 15 μ. The motor branches also showed a peak in the 3 μ group, but there was in addition a second pronounced peak in the 15 μ group. This distribution is in fact more strongly bimodal than that found by Gutmann & Sanders [1943] for the mixed part of the peroneal nerve. The second population shown on the bimodal curve, which has its mean at 15 μ, presumably contains the alpha fibres of the A group.



Text-fig. 1. Histograms to show the normal fibre-size spectrum in the 'motor' and 'cutaneous' branches of the peroneal and tibial nerves, and in the posterior tibial nerve. Rabbit 928.

From the above data it is clear that marked differences exist both in the size of the largest fibres, and in the form of the distribution curve of fibre sizes, between the motor and cutaneous divisions of both the peroneal and tibial nerves.

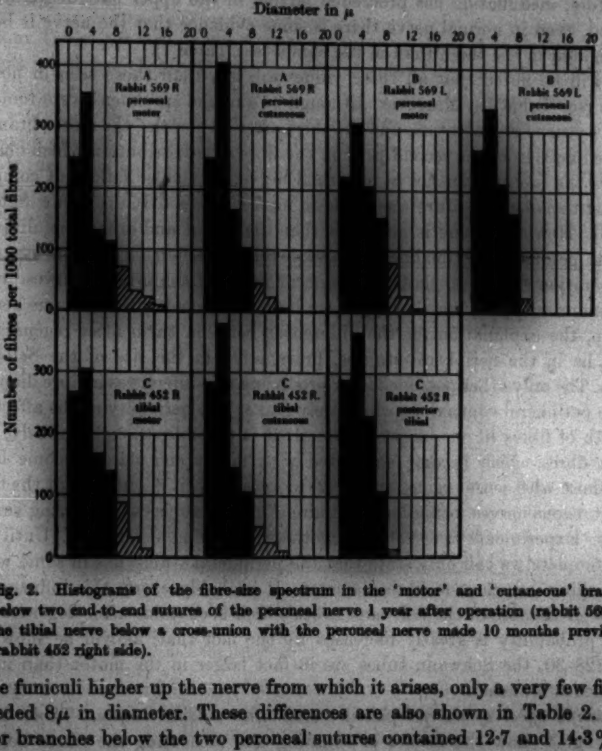
Regeneration after nerve suture of larger fibres in motor, smaller fibres in cutaneous branches. Fibres were counted and measured in the two divisions of the peroneal nerve below two direct end-to-end sutures of the nerve made 1 year previously, and below a cross-union of the central stump of the cut peroneal nerve with the peripheral stump of the tibial made 10 months previously. Table 2 and Text-fig. 2 show the results of these measurements.

TABLE 2. Numbers and sizes of fibres in the 'motor' and 'cutaneous' divisions of the nerve. (A) and (B) below sutures of the peroneal nerve made 1 year previously. Left and right sides of rabbit 569. (C) in the tibial peripheral stump below a peroneal-tibial cross-union made 10 months previously. Right side of rabbit 452

	Nerve	Total no. of fibres	No. of fibres larger than 8 μ	% large fibres	Average-fibre diameter in μ
(A) Peroneal 569 R	Motor	4,380	556	12·7	4·25
	Cutaneous	2,743	244	8·9	3·78
	Total	7,123			
(B) Peroneal 569 L	Motor	4,612	660	14·3	4·31
	Cutaneous	2,636	190	7·2	3·72
	Total	7,248			
(C) Tibial in thigh 452 R	Motor	4,970	549	11·0	4·31
	Cutaneous	7,155	165	2·3	3·67
	Total	12,125			
Posterior tibial in shank 452 R		4,505	65	1·4	3·39

All of the branches contained somewhat fewer fibres than are found in normal nerves. However, the ratio of the total number of fibres in the motor and cutaneous divisions is approximately normal. This in itself indicates that some factor in the peripheral stump controls the number of fibres which regenerate there. The fibres were also counted in the central stumps above the two peroneal sutures, and in the peroneal nerve above the cross-union. Suture A (see Table 2) had a central stump containing 9026 fibres, while the central stump above suture B contained 9813 fibres. Therefore the peripheral stumps in these cases contained respectively 79 and 74% of the number of fibres in the corresponding central stumps. By contrast, below the cross-union there were 12,125 fibres, 180% of the number present in the central stump (6724). This considerable excess of fibres in the periphery indicates that the central fibres have undergone branching to supply an increased peripheral demand, a fact already observed by many authors [see Aird & Naffziger, 1939, for summary]. Even so, the tibial peripheral stump in this animal contained fewer fibres than the normal tibial nerve (see Table 1).

Text-fig. 2 is a plot of the frequency distribution of fibre sizes in the two parts of these regenerated nerves, expressed as per 1000 of those measured. In all cases the motor branches contained larger fibres than the cutaneous nerves (see Pl. 1, figs. 3, 4). The two peroneal sutures had motor funiculi containing fibres up to 18μ in diameter, while those in the cutaneous part did not exceed 14μ. Below the cross-union the fibres were smaller than after direct peroneal suture. This very interesting observation would be explained on the assumption that the increase in diameter of a fibre depends on the supply of axoplasm which it obtains by outflow from the central stump [see Young, 1942; Gutmann & Sanders, 1943]. Since the cross-union involves the formation of many branches, each central fibre has to provide for a greater number of peripheral fibres than after direct suture. The maximum size in the motor branches below the cross-union was 14μ, while in the posterior tibial or the funiculi higher up the nerve from which it arises, only a very few fibres exceeded 8μ in diameter. These differences are also shown in Table 2. The motor branches below the two peroneal sutures contained 12·7 and 14·3% of fibres larger than 8μ, while in the cutaneous branches there were only 8·9 and 7·2% of large fibres. The average fibre diameters were also larger in the motor branches. Below the cross-union the contrast between the two sets of branches was even more striking; the motor branches contained 11·0% of large fibres, and had an average-fibre diameter of 4·31μ; the cutaneous funiculi contained only 2·3% of larger fibres and had an average-fibre diameter of only 3·67μ.



Text-fig. 2. Histograms of the fibre-size spectrum in the 'motor' and 'cutaneous' branches below two end-to-end sutures of the peroneal nerve 1 year after operation (rabbit 569); in the tibial nerve below a cross-union with the peroneal nerve made 10 months previously (rabbit 452 right side).

In the posterior tibial nerve the proportion was still lower, there being 1·4% of larger fibres, and an average-fibre diameter of only 3·39 μ. The total number of medullated fibres is also very much lower in this nerve in the shank than in the large funiculus in the thigh which gives rise to it. Careful dissections of the nerve behind the gastrocnemius muscle have failed to show any branch arising from this funiculus before it becomes the posterior tibial. Evidently, therefore, medullation has proceeded farther in the upper part of the nerve. Indeed, even in normal nerve there is some evidence that the nerve is better myelinated in the thigh than in the shank (see Table 1).

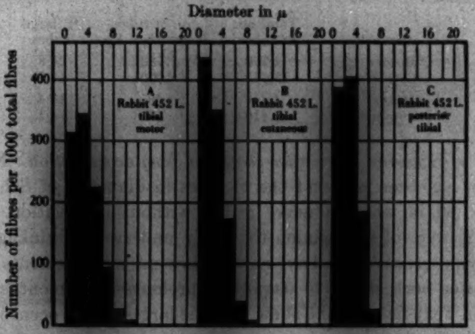
It will be noted that all these values are less than those seen in normal nerve (see Table 1). Moreover, although two of the specimens were removed after 1 year of regeneration, and the third after 10 months, the motor branches showed no sign of the second peak in the fibre size distribution which characterizes the motor branches of a normal nerve. A similar failure of complete reconstitution after suture was noted in mixed nerves by Gutmann & Sanders [1943]. However, there is no doubt that the motor and cutaneous divisions of both peroneal and tibial nerves have developed differences in fibre size after regeneration similar to those which occur between the normal nerves. Since the motor and cutaneous divisions are both connected with the same central stump, the explanation for the differences between them after regeneration must lie in the peripheral stumps, presumably in the size of the Schwann tubes. The only other possible factor producing the differences in fibre diameter is the peripheral connexions which they make. The periphery might affect the growth of fibres in one of two ways: (1) it is possible, though unlikely, that those fibres which become connected with motor end-plates become large, and those with sense organs, small; (2) more simply, it might be that the tubes of cutaneous nerves, being longer, drain off more axoplasm, producing smaller fibres. Experiments to test these hypotheses are being undertaken. Until they are complete we can only state that the peripheral stump has in some way a controlling influence on fibre diameter. It probably exerts this influence by virtue of the size of the Schwann tubes which it contains after degeneration. This probability is greatly increased by the fact that, as will be shown on pp. 128–30, the Schwann tubes are in fact larger in the motor than in the cutaneous branches.

Specific effect of the peripheral stump after delayed suture. Table 3 and Text-fig. 3 show the results of counting and measuring the fibres below a cross-union 10 months after the central end of the peroneal nerve had been joined into a tibial peripheral stump which had previously been degenerating for 15½ months. Comparison of the fibre sizes in the motor branches with those in the cutaneous division showed that the specific power of the peripheral stump to produce fibres of different sizes in the two divisions is retained even after so long a period of degeneration (see also Pl. 1, figs. 5, 6).

TABLE 3. Numbers and sizes of fibres in the divisions of the tibial nerve after 15½ months of denervation followed by 10 months of reinnervation by cross-union with a peroneal central stump (rabbit 452 left side)

Nerve	Total no. of fibres	No. of fibres larger than 8 μ	% large fibres	Average-fibre diameter in μ
Motor	3742	102	2·7	3·36
Cutaneous	5529	23	0·4	2·65
Total	9271			
Posterior tibial	2165	1	0·05	2·70

From Table 3 it will be seen that the total number of medullated fibres present in the peripheral stump was 9271, that is to say, somewhat less than was found in the tibial nerve below a similar cross-union made immediately after section of the tibial (Table 2), which was in fact on the opposite side of the *same animal*. Longitudinal sections showed equally close apposition at both suture lines, so that the deficiency of fibre size and medullation after the secondary suture is not due to bad union of the stumps. The relative number of fibres in the two divisions was approximately the same as in the immediate cross-union. However, in spite of great delay of suture, there was still an excess of fibres in the periphery compared with the number in the central stump, which contained 6677 fibres. Therefore branching of fibres takes place even when the peripheral stump has been long denervated. The posterior tibial nerve contained only 2165 fibres, less than half the number present at this level below the immediate union on the opposite side, and less than a third the number present in the normal nerve (see Table 1).



Text-fig. 3. Histograms of the fibre-size spectrum 10 months after a cross-union of the central stump of the peroneal with the peripheral stump of the tibial nerve previously degenerated for 15½ months. (A) in the 'motor' branches; (B) in the 'cutaneous' branches; (C) in the posterior tibial nerve (rabbit 452 left side).

In the motor branches there were 102 fibres larger than 8 μ, 2·7 % of the total number present. These were many fewer than in the normal nerve (36·8 %), or below the immediate cross-union on the opposite side (11%), but more than in the cutaneous funiculi, which contained only twenty-three large fibres (0·4%). The posterior tibial nerve contained only a single fibre larger than 8 μ. The average-fibre diameters showed similar differences, the value for the motor branches (3·36 μ) exceeding that for the cutaneous division (2·65 μ). These values are much less than those recorded 10 months after an immediate cross-union of the tibial and peroneal nerves. As would be expected, the histogram of fibre sizes in the motor funiculi shows no trace of bimodality.

These results indicate that while the fibres are much fewer and smaller in all branches of the nerve after a long delayed than after an immediate re-innervation, the specific differences between the motor and cutaneous branches are retained. The simplest explanation of this difference is that the Schwann tubes in the peripheral stump retain their specific differences in diameter, even during the later stages of degeneration.

TABLE 4. Numbers and diameters of Schwann tubes in the divisions of tibial nerves degenerated for (A) 25 days (rabbit 724) and (B) 15¼ months (rabbit 722)

Time	Branch	Total no. of tubes	No. of tubes larger than 8 μ	% large tubes	Average-tube diameter in μ
25 days	Motor	4,770	1183	24·8	6·25
	Cutaneous	13,675	1600	11·7	4·48
462 days	Motor	4,516	605	13·4	4·03
	Cutaneous	11,410	468	4·1	3·49

Diameters of Schwann tubes after short and long degeneration. No thorough investigation has been made of the changes in diameter of Schwann tubes during a period in which a nerve is left without innervation. Holmes & Young [1942] were of the opinion that they underwent shrinkage, and that it was slowly progressive. We have now made some measurements of Schwann tube sizes after both short (25 days) and prolonged (18 months) periods of degeneration. Text-figs. 4 and 5 show that the largest tubes occur in the motor branches in both cases.

Text-fig. 4 shows the distribution of tube sizes per 1000 total tubes in the motor branches of the tibial nerve after 25 days' degeneration. It will be seen that most of the tubes occur in the 0–2 μ group, so that the diagram at first sight appears strikingly different from the histogram of the normal spectrum of the fibre diameters (see Text-fig. 1). It must be remembered, however, that when studying a normal nerve we measure only those fibres which stain with a method designed to demonstrate the myelin sheath, whereas in this case the method stains the collagenous tubes investing the nerve fibres. In the case of tube measurements we are no doubt including in the smallest size group the tubes of many non-myelinated fibres. Accordingly, to make the histograms of tube size more closely comparable with those obtained by measuring nerve fibres, the actual value for the number of tubes in the smallest group was in each case replaced by the number of myelinated fibres found in this group in normal nerve. This is of course a purely arbitrary procedure, particularly in the case of the long-denervated stump, but the proportion of tubes larger than 2 μ so obtained are of the same order as those

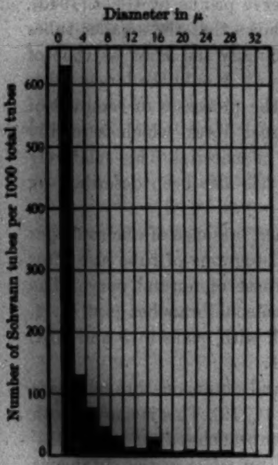


Fig. 4.

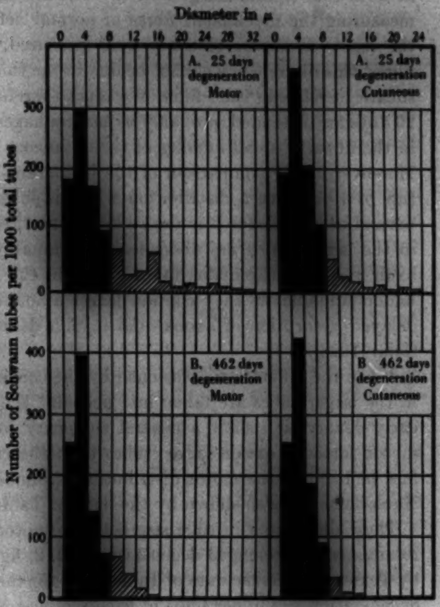


Fig. 5.

Text-fig. 4. Histogram of the spectrum of sizes of the Schwann tubes in the 'motor' branches of the peripheral stump of the peroneal nerve 25 days after section. The figure is not corrected for the presence of unmyelinated fibres (rabbit 724).

Text-fig. 5. Corrected histograms (see text) of the spectrum of Schwann-tube sizes in the 'motor' and 'cutaneous' branches of the peripheral stump of the tibial nerve after degeneration for (A) 25 days, (B) 462 days.

found when measuring nerve fibres. Obviously, however, the numbers in the size group 0–2 μ in the histograms of tube-size spectra are without significance. Moreover, the values of the various factors in Table 4, which were calculated after assigning this arbitrary value to the number of tubes in the 0–2 μ group, cannot be directly compared with the values of these factors in the other tables.

The corrected histograms of tube size obtained in this way are given in Text-fig. 5. After 25 days' denervation the cutaneous branches contained tubes up to 24 μ in diameter, while in the motor branches the upper limit of tube size was 32 μ. It will be noted at once that the upper limit of tube diameter in both sets of funiculi greatly exceeds the largest diameter recorded when measuring the myelinated fibres of normal nerve (cf. Text-figs. 1, 4). Since the *inside* diameters of tubes were measured, and the *outside* diameters of fibres, this discrepancy cannot be due to the thickness of the connective-tissue sheath enveloping each fibre. We must therefore conclude that the tubes at 25 days are distended, probably by macrophages, an appearance already noted in the fibres of small bundles of the cutaneous nerve plexus by Glees [1943]. Moreover, closer comparison of the histograms shows that the smaller tubes (up to 10 μ) have a distribution which is substantially the same as that of normal nerve. Hence it is probable that at 25 days it is only the larger tubes in the nerve which are distended. Presumably the processes of removal of axon and myelin remains takes longer in the larger fibres, which is readily understandable, as they contain larger amounts of material. This relatively delayed absorption of axonal and myelin debris has also been described by Cajal [1928].

When the differences occasioned by the continued presence of macrophages in the larger tubes are taken into account, the histograms of tube size after early denervation show a general similarity to those of normal nerve. The motor funiculi contain larger tubes than the cutaneous branches (see Pl. 2, figs. 9, 10), and the motor distribution is bimodal, with peaks at 3 and 15 μ. These facts are also shown by the data of Table 4.

These differences in tube size between motor and cutaneous branches are retained after prolonged degeneration (Pl. 2, figs. 11, 12). This is shown both by Text-fig. 5 and the data of Table 4. In the motor branches after 18 months' degeneration the upper limit of tube size was at 18 μ while in the cutaneous branches it was at 14 μ, there being very few tubes, however, greater than 12 μ. Table 4 shows that the percentage of large tubes, and the average-tube size, were larger in the motor funiculi. Moreover, this table also shows that there are fewer large tubes, and the average tube sizes are smaller, after a long than after a short period of denervation. In fact, after the long period the percentages of large *tubes*, and the average *tube sizes* of both divisions of the nerve, are markedly less than the percentages of large *fibres*, and the average *fibre sizes* of normal nerve (cf. Table 1).

The histogram of tube sizes in the motor division of the stump denervated for a longer time does not show the bimodality that is characteristic of the same branches after only 25 days of denervation. If the only change in the diameter of the tubes during denervation were a progressive shrinkage, we should expect this bimodality to be retained, even though the second peak in the distribution curve would appear at a lower diameter. However, the histogram shows a plateau between 6 and 10μ which gives it a very different shape from that in the cutaneous branches. Until there is more information about the time of the disappearance of the bimodality of this distribution it is not possible to say more as to its cause.

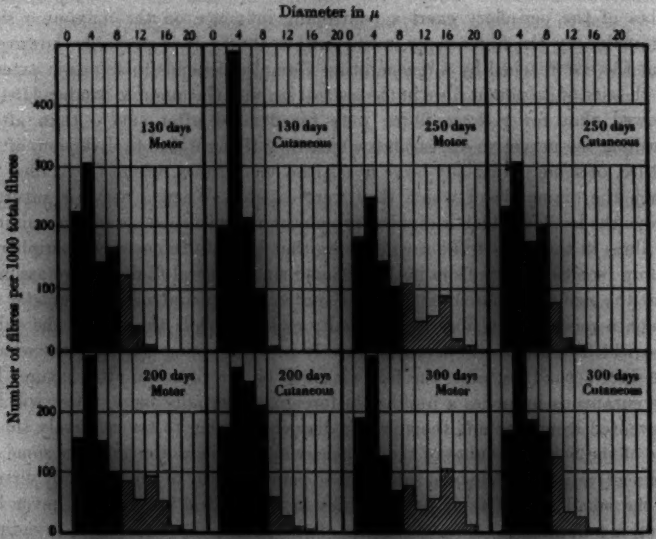
It is clear from the above data that the differences in tube size after different times of denervation parallel the differences between the fibre spectra achieved after immediate and delayed suture. Specificity is retained, but both the tubes and the fibres which regenerate in them are smaller after long delay. We are thus led to conclude that the sizes attained by nerve fibres during regeneration are at least partly determined by the size of the tubes into which the fibres penetrate.

Fibre sizes in the different branches after crushing the nerve. The results of the experiments described above give us reason to believe that the Schwann tubes of the periphery exert a controlling influence on the maximum size attainable by the fibres regenerating within them. It is probable, however, that the size reached by a regenerating fibre also depends to a certain extent on the size of its parent fibre in the central stump. Gutmann & Sanders [1943] showed that fewer large fibres were regenerated after suture than after crushing the nerve, an appearance which they thought due to diversion, after suture, of some potentially large fibres into small tubes, where they failed to reach the diameters of which they were capable. When a nerve is sutured above the level at which it divides into motor and cutaneous branches, mixing of fibres in the scar of union will ensure that both divisions of the peripheral stump receive sprouts from both large and small central fibres. If we assume that the size of its parent fibre in the central stump controls the diameter of which a regenerating fibre is potentially capable, while the size of the tube into which it penetrates acts restrictively in determining the diameter which the fibre actually attains, the following will be the situation after suture: In the cutaneous branches a number of foreign, potentially large, fibres will be prevented from reaching their proper diameters owing to the relatively small size of the Schwann tubes of the branch; while in the motor branches some of the relatively large Schwann tubes will be penetrated by fibres, which, by virtue of the small size of their parent fibres in the central stump, can never be anything but small. However, some of the potentially large fibres invading the motor branch will be in large tubes and thus able to reach their proper diameters, while all similar fibres in the cutaneous branches will fail to do so. Thus the net result of regeneration after suture will be to produce larger fibres in the motor than in the cutaneous branches, although the difference in mean diameter between the two sets of funiculi will be less marked than in normal nerve. That this is indeed the case can be seen from a comparison of Table 1 with Tables 2 and 3.

A further test of this hypothesis is provided by the conditions obtaining after interruption of the nerve by crushing. Under these conditions very little mixing of fibres in the scar occurs. Therefore potentially large fibres will tend to enter large tubes, and vice versa. Regeneration after crushing should thus produce greater differences in mean fibre diameter between the cutaneous and motor branches than after suture; the distribution in the motor branches should eventually become bimodal, and, in later regenerates, the values

TABLE 5. Numbers and sizes of fibres in the motor and cutaneous divisions of the peroneal nerve at different times after crushing the nerve

Time	Branch	Total no. of fibres	No. of fibres larger than 8 μ	% large fibres	Average-fibre diameter in μ
130 days (rabbit 702)	Motor	4844	799	16·5	4·58
	Cutaneous	3500	7	0·2	3·42
200 days (rabbit 706)	Motor	4912	1464	29·8	6·07
	Cutaneous	3880	357	9·2	4·64
250 days (rabbit 715)	Motor	4176	1336	32·0	6·31
	Cutaneous	3035	240	7·9	4·23
300 days (rabbit 704)	Motor	4258	1409	33·1	6·52
	Cutaneous	2548	471	18·5	4·98



Text-fig. 6. Histograms of the spectrum of sizes of myelinated nerve fibres in the 'motor' and 'cutaneous' branches of the peroneal nerve at different times after crushing the nerve.

assumed by various factors should approach those for normal nerve. Fibres were therefore counted and measured in the motor and cutaneous divisions of the peroneal nerve, 130, 200, 250 and 300 days after this nerve had been interrupted by firm localized crushing with smooth-tipped forceps. The results of these measurements are given in Table 5 and Text-fig. 6.

The results show that, even as early as 130 days after crushing, the fibres in the motor funiculi are larger than in the cutaneous bundles. The percentages of large fibres and the average-fibre diameters are, however, smaller than the normal value in both divisions, and the histogram of fibre distribution in the motor division shows no secondary peak. At 200, 250 and 300 days after crushing the large-fibre peak at 15 μ is present. Moreover, in the motor branches the value of the large-fibre percentage and the average-fibre diameter approach those of normal nerve (cf. Table 1). However, in the cutaneous funiculi it is only at 300 days that the large-fibre percentage approaches closely to the normal value, although the average-fibre diameters at all three times have a value similar to normal.

These figures indicate that, after crushing, the nerve becomes very much more fully reconstituted than after suture, even the fibre spectra being eventually restored (see also Pl. 1, figs. 7, 8). Moreover, the difference in average-fibre diameter between the cutaneous and motor branches finally becomes as great as normal (cf. Tables 1, 5).

DISCUSSION

From the results of these experiments we may conclude that the final pattern of fibre sizes achieved after the regeneration of a nerve is determined by at least two factors. First, the final diameter of which any regenerating fibre is potentially capable depends on the calibre of its parent fibre in the central stump. Secondly, the maximum diameter which any fibre actually attains in the peripheral stump depends on the size of the particular Schwann tube into which it penetrates. Thus if a stream of outflowing axoplasm whose parent fibre is large finds its way into a large tube the resultant mature fibre is large, but if the tube receiving the outflow is small, the final size which the fibre attains is limited by the tube and only a small fibre results. Conversely, if the outflow from a small parent fibre penetrates a large tube, the fibre remains small, as it cannot achieve maturation up to the limits of the tube.

The peripheral stump preserves the pattern of the nerve during degeneration. Parts of the nerve which normally contain fibres of definite and specific sizes retain their specificity even during prolonged degeneration. However, long-term denervation is accompanied by shrinkage of peripheral Schwann tubes, and it is doubtful whether the deficiencies in fibre diameter which will result from this process when the stump becomes reinnervated can ever be made up. Holmes & Young [1942] found that 3 months after a delayed suture the diameter of fibres and degree of medullation in the peripheral stump were smaller than after immediate suture. The present experiments show that this deficiency continues to be manifest even after 10 months of regeneration. Comparison of figs. 13 and 14 of Pl. 2 and Text-figs. 2 and 3 shows that the growth of fibres and their medullation has proceeded much less far on the side with the delayed suture. The degree of difference between the medullation of the two sides is not obviously less than after the 3 months' regeneration studied by Holmes & Young [1942]. If there is any catching up on the part of the long denervated side it must be a very slow process. Since deficiency of medullation can hardly fail to have an adverse effect on the efficiency of functioning of nerves, we must further emphasize the conclusion that prolonged delay in suturing prejudices the chances of good recovery. This has often been suggested by clinicians [see Foerster, 1929], and it is now apparent that many peripheral factors are likely to contribute to it [see Gutmann & Young, 1944] in addition to the actual making of the union [Holmes & Young, 1942], and the deficiency in medullation here reported. Unfortunately, it is not yet possible to say exactly to what extent the various factors operate with given delay in suturing. In particular, we cannot yet plot the curve of shrinkage in diameter of Schwann tubes during the months of degeneration. But it seems likely that any delay beyond the first month will adversely affect functional recovery. Apart from certain special factors which operate during the first month of degeneration, we may say that every delay puts further obstacles in the way of the process of regeneration.

SUMMARY

1. In the lower part of the thigh of the rabbit the peroneal and tibial nerves divide into two sets of funiculi: (*a*) those which supply the extensor and flexor muscles of the shank; and (*b*) those which give rise to the anterior and posterior tibial nerves and are predominantly sensory and sympathetic. Counts and measurements of the medullated nerve fibres present in these two situations show that the motor branches contain larger fibres than those which give rise to the anterior and posterior tibial nerves (cutaneous). Moreover, the frequency distribution of fibre diameters in the motor funiculi is bimodal, with peaks at 3 and 15μ.

2. One year after end-to-end suture of the peroneal nerve, and 10 months after cross-union of the peroneal to the peripheral stump of the tibial nerve, the motor funiculi were found to contain larger fibres than the cutaneous ones. The largest fibres in both branches were smaller than in normal nerve, and the frequency distribution of fibre diameters in the motor funiculi was not bimodal.

3. In a case in which the peroneal nerve was cross-united to a tibial peripheral stump, which had been previously denervated for 18 months, larger

fibres were found, after 10 months of regeneration, in the motor funiculi than in the cutaneous. Fibres in both sets of funiculi were, however, smaller than after immediate cross-union, and the frequency distribution of fibre diameters in the motor funiculi was not bimodal.

4. After 25 days of denervation the Schwann tubes of the motor funiculi were found to be larger than those of the cutaneous, and to have a bimodal distribution. Even after 15½ months of denervation, larger tubes were still found in the motor funiculi, but the tubes throughout the nerve were smaller than after only 25 days' denervation.

5. After crushing, the peroneal nerve regenerated larger fibres in the motor than in the cutaneous funiculi. The nerve was, however, much more completely reconstituted after crushing than after suture or cross-union. Even the bimodal character of the frequency distribution in the muscular branches was completely restored.

6. All these results indicate that the Schwann tubes which replace degenerating nerve fibres in the peripheral stump preserve the pattern of fibre sizes in the periphery during degeneration, and control the pattern of fibre sizes which appears as a result of regeneration. This they do by restricting the diameter attained by new fibres regenerating within them. In the absence of this restrictive influence, the diameter of which a regenerating fibre is potentially capable depends upon the calibre of the fibre from which it arises in the central stump.

7. Since the Schwann tubes shrink during prolonged degeneration, delay of suture is followed by the regeneration of fibres which are abnormally small and poorly medullated.

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EXPLANATION OF PLATES 1 AND 2

All photographs showing myelinated nerve fibres were made from preparations stained by the Flemming-Weigert technique. Figs. 9–12 are of Schwann tubes stained with Heidenhain's picro-blue-black.

Plate 1

Fig. 1. Myelinated nerve fibres in a motor funiculus of the normal tibial nerve. (928*a* 1. 4.)

Fig. 2. Myelinated nerve fibres in the funiculus of the normal tibial nerve which gives rise to the posterior tibial nerve. Note that the fibres are smaller than in fig. 1. (928*a* 1. 4.)

Fig. 3. Myelinated nerve fibres in a motor funiculus below an end-to-end suture of the peroneal nerve made 1 year previously. (569*h* 2. 3.)

Fig. 4. Myelinated nerve fibres in a cutaneous branch below an end-to-end suture of the peroneal nerve made 1 year previously. Note smaller fibres than in fig. 3. (569*h* 2. 3.)

Fig. 5. Myelinated nerve fibres in a motor funiculus of the tibial nerve after 15½ months of degeneration followed by 10 months of reinnervation. (452*g* 7. 4.)

Fig. 6. Myelinated nerve fibres in a cutaneous branch of the tibial nerve after 15½ months of degeneration followed by 10 months of reinnervation. Note smaller fibres than in fig. 5. (452*g* 7. 4.)

Fig. 7. Myelinated nerve fibres in a motor funiculus 300 days after crushing the peroneal nerve. (704*e* 1. 2.)

Fig. 8. Myelinated nerve fibres in a cutaneous branch 300 days after crushing the peroneal nerve. Note smaller fibres than in fig. 7. (704*e* 1. 2.)

Plate 2

Fig. 9. Schwann tubes in a motor funiculus of the peripheral stump of the tibial nerve 25 days after section. (724*c* 3. 1.)

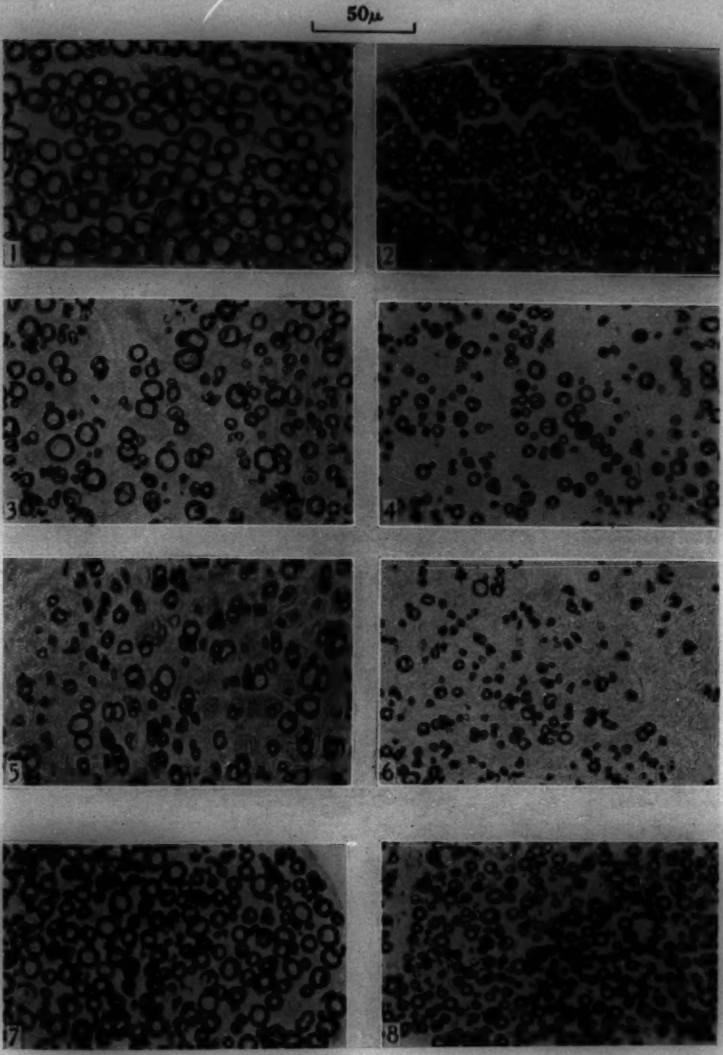
Fig. 10. Schwann tubes in a cutaneous branch of the peripheral stump of the tibial nerve 25 days after nerve section. Note smaller tubes than in fig. 9. (724*c* 3. 1.)

Fig. 11. Schwann tubes in a motor funiculus of the peripheral stump of the tibial nerve 15½ months after nerve section. (722*a* 1. 1.)

Fig. 12. Schwann tubes in the cutaneous branch of the peripheral stump of the tibial nerve 15½ months after nerve section. Note smaller tubes than in fig. 11. (722*a* 2. 2.)

Fig. 13. Myelinated nerve fibres in the posterior tibial nerve 55 mm. below an immediate cross union with the central stump of the peroneal nerve made 10 months previously. (452*h* 7. 4.)

Fig. 14. Myelinated nerve fibres in the posterior tibial nerve 10 months after operation, 55 mm. below a cross union of the central stump of the peroneal nerve with the peripheral stump of the tibial nerve sectioned 15½ months before the union was made. Note smaller and fewer fibres than in fig. 13. (452*a* 7. 4.)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Figs. 1–8.

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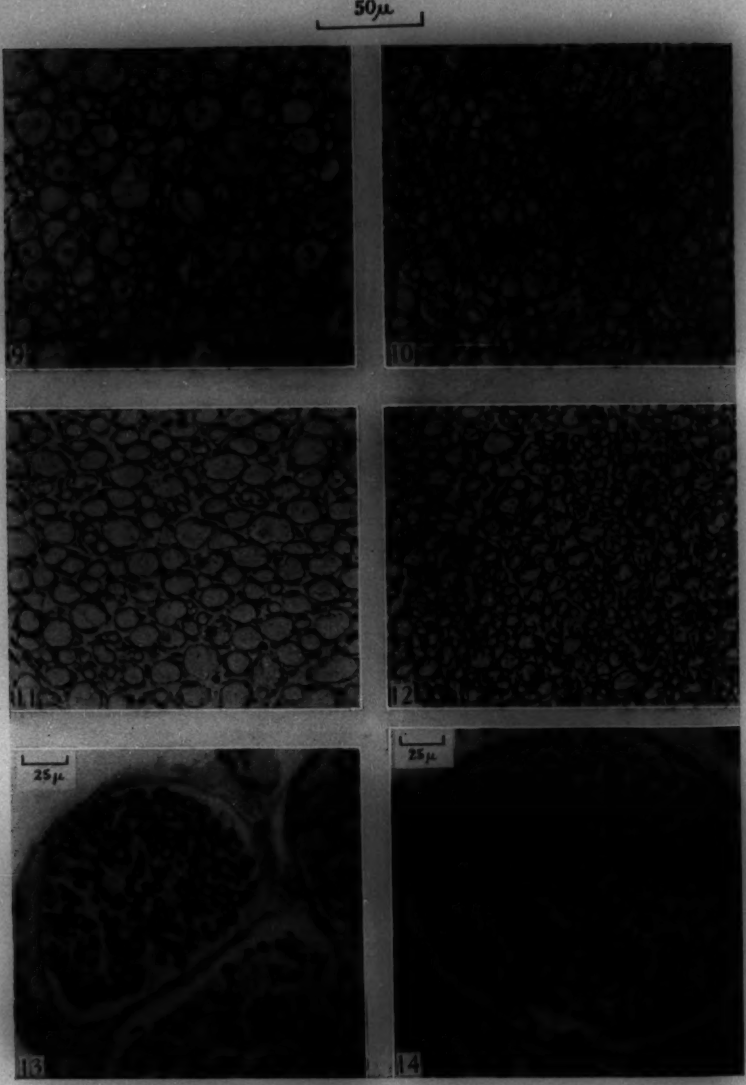
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THE EFFECT OF MORPHINE AND HYOSCINE ON DYE CONCENTRATION CURVES IN PLASMA VOLUME DETERMINATION

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(*Received* 13 *August* 1943)

When Evans blue is injected into the circulation of normal individuals a constant dye concentration is reached within 20 min. and this concentration is maintained for a further 40 min. Subsequently it diminishes at a rate of about 5 % per hour [Crooke & Morris, 1942]. In the course of investigations upon the plasma volume in patients with shock, however, unaccountable alterations in the concentration of dye were sometimes found. A systematic examination of the factors which might be responsible for such anomalous dye concentration curves has therefore been made.

At first the anomalous results were thought to be associated with shock, but this was disproved by a normal curve occurring in a patient with severe traumatic shock, and whose systolic blood pressure was consistently below 60 mm. Hg (Fig. 1*a*). In contrast to this, a moderately shocked patient with second and third degree burns of the hands and face, whose systolic blood pressure varied from 140 to 150 mm. Hg, had an anomalous curve (Fig. 1*b*).

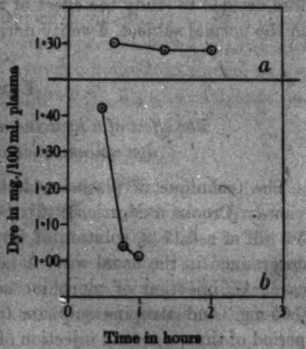

Fig. 1. Dye concentration curves in shock. Injection of dye at 0 hours. 35 mg. injected.

In order to investigate the problem under more controlled conditions, the dye concentration curves were examined in fourteen patients undergoing various major operations. The patients were given an injection of dye before the operation and the concentration curve determined throughout the operation. A second injection of dye was then given in order to correct for the loss of dye from the circulation since the first injection, and the concentration curve again determined. About half of the dye concentration curves so obtained were anomalous. Anomalous second curves were not related to the second injection of Evans blue because we found that when two successive injections were given to normal individuals and correction made for the dye already in the circulation, normal flat dye concentration curves were obtained (Fig. 2).

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[1] Freedom Research Fellow.

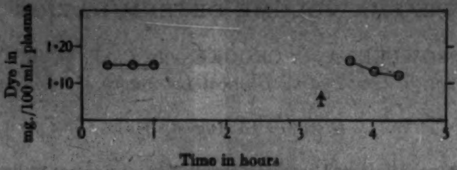

Fig. 2. Dye concentration curves in a normal subject. First injection of dye at 0 hours (35 mg.). Second injection of dye at the arrow (35 mg.).

The anomaly was also found to be unrelated to the type or severity of the operation. Nor was the anaesthetic responsible for it, because an anomalous curve began in one patient before the anaesthetic was started, and in another a normal curve was obtained immediately after a long period of anaesthesia. In all cases a hyoscine-morphine-atropine mixture had been given and in some cases the injection of these drugs preceded the injection of dye. It was therefore decided to study the effect of these drugs on the dye concentration curve in the normal subject. Twenty normal volunteers were examined.

EXPERIMENTAL

The effect of a hyoscine-morphine-atropine mixture on the dye concentration curve in the normal subject

The technique of plasma volume determination has been described previously [Crooke & Morris, 1942]. Seven normal volunteers were injected with 5·0 ml. of a 0·17 % solution of Evans blue and the dye concentration curve determined in the usual way. A normal control curve was obtained in every case. An injection of morphine sulphate 16·2 mg., hyoscine hydrobromide 0·65 mg., and atropine sulphate 0·65 mg. was then given. After a variable period of time, a second injection of the same amount of Evans blue was given and the dye concentration curve again determined. Generally the curve showed a preliminary fall followed by a rise which was most marked 1–1·5 hr. after the second injection of dye. There was then a second fall which ultimately flattened out at a level significantly lower than that of the control curve. This type of curve is illustrated by Fig. 3*b*. It is very similar to that shown by the patient with burns (Fig. 1*b*), who had received an injection of morphine sulphate 90 min. before the injection of Evans blue. Individual variations in the shape of the second curve were sometimes observed (Fig. 3*a*, *b*).

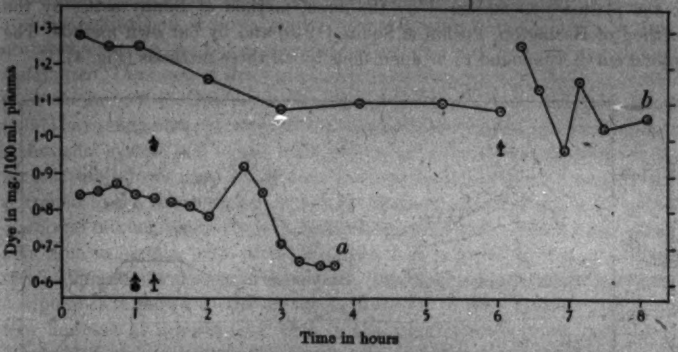

Fig. 3. Anomalous curves in normal subjects. *a*, after morphine. *b*, after morphine-hyoscine-atropine mixture. First injection of dye at 0 hours (35 mg.). Injection of drug at ⇑. Second injection of dye at ↑ (35 mg.).

The individual effects of morphine, hyoscine and atropine on the dye concentration curve

The course of these experiments was similar to that described above, the drugs being administered separately. Eight normal volunteers were examined; three with morphine sulphate, two with hyoscine hydrobromide and three with atropine sulphate. Morphine sulphate and hyoscine hydrobromide had similar actions and typical anomalous curves were produced. Atropine sulphate had but little effect.

The persistence of the effect in the normal subject

A series of experiments was carried out in order to establish the duration of the effect in the normal subject. In one volunteer an anomalous curve was observed 5 hr. after the injection of morphine sulphate. This is seen in Fig. 3*b*, which also shows that when the dye is injected before the drug a normal curve is obtained for 5 hr. after the administration of the drug. This effect was observed invariably, even when only 10 min. had elapsed between the injection of the dye and the drug.

Simultaneous studies of the effect by different methods of plasma volume estimation

The possibility that the anomaly was due to the method of estimating Evans blue in plasma was suggested by the work of Bonnycastle [1942]. He used the direct method of estimation and found no alteration in plasma volume following the injection of morphine. A healthy fasting volunteer was therefore subjected to the experimental procedure already described, an injection of the morphine-hyoscine-atropine mixture being given. The dye concentration in the plasma was measured by the direct method of Bonnycastle, by the method of Harington, Pochin & Squire [1940] and by our own method. The second curve was found to be anomalous by all three methods (Fig. 4).

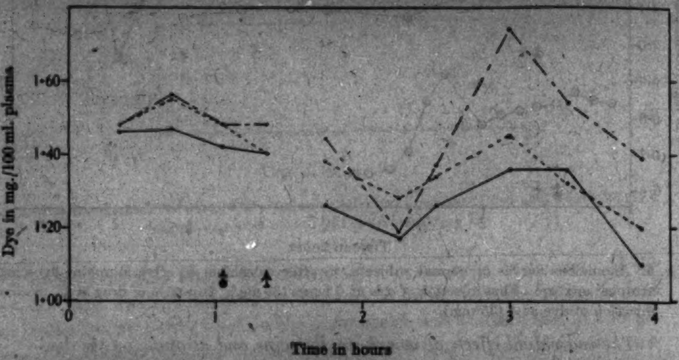

Fig. 4. Anomalous curves in a normal subject. Plasma volume estimated by three methods. —— Crooke & Morris [1942]. ---- Bonnycastle [1942]. —·— Harington, Pochin & Squire [1940]. First injection of dye at 0 hours (35 mg.). Injection of drug at ↕. Second injection of dye at ↑ (35 mg.).

DISCUSSION

It is probable that at least two factors are concerned in the production of anomalous dye concentration curves. The first is unequal distribution of Evans blue in the circulation. This is suggested by the initial fall and subsequent rise which occurs in most anomalous curves. The delayed return of dye to the peripheral circulation can hardly be explained by any hypothesis other than that of unequal distribution. Rein [1929] has shown that the partition of blood between the peripheral and visceral regions of the circulation can vary widely in dogs with small alterations in temperature within the physiological range, and also under the influence of certain drugs. Morphine and hyoscine may influence the distribution of blood to different parts of the circulation. The small volume of blood in the antecubital vein containing the whole of the injected dye will then be distributed according to the rate of flow in the different parts of the circulation, those areas with the greater perfusion rate receiving the larger quantity of dye. Eventually the distribution will become uniform. The second factor is an abnormally rapid elimination of Evans blue from the circulation. The amount of dye lost varies markedly in different subjects, but it has never exceeded the amount given at the second injection. After the period of abnormally rapid elimination the dye concentration again becomes relatively constant. This may be obscured however in subjects showing the most rapid elimination because the concentration then becomes so low that accurate estimation is impossible. Abnormally rapid elimination has never occurred when the dye was given before the drug so that the dye was uniformly distributed. The cause of this abnormally rapid elimination of dye is uncertain, since the normal mechanism of elimination is still obscure. Gibson & Evans [1937] have suggested that dyes of this class are mainly taken up by the reticulo-endothelial system and stored there until their eventual degradation. In the normal subject we have found that the rate of elimination is roughly proportional to the concentration of dye in the blood. If therefore, as a result of abnormal mixing, relatively high concentrations of dye persist for an appreciable time in certain parts of the circulation, it is probable that abnormally rapid elimination will occur in these areas. Probably mixing eventually becomes complete as is shown by the flattening of the curve. The dye concentration is now however at a lower level than in the initial control curve, indicating a falsely high plasma volume. This explanation of abnormally rapid elimination of dye which occurs only during the period of abnormal mixing is consistent with the observation that dye which is already normally mixed prior to the injection of the drug is eliminated at the normal rate.

Normal flat curves are invariably obtained if the injection of Evans blue precedes the injection of morphine or hyoscine by a period (about 20 min.) sufficiently long to allow of normal mixing. Plasma volume may be determined satisfactorily in shocked patients provided this precaution is observed.

SUMMARY

1. Anomalous dye concentration curves have been found in plasma volume determinations following the administration of morphine and hyoscine.

2. The mechanism responsible for this anomaly is discussed.

The authors are indebted to Dr Miles for access to clinical material at Oldchurch Hospital, Romford, and to Mr R. Wilton for technical assistance. The cost of this research was defrayed from the Yarrow Fund of the London Hospital.

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THE BEHAVIOUR OF THE CERVIX UTERI *IN VIVO*

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(*Received* 23 *October* 1943)

The most important work on the behaviour of the cervix uteri is that of Newton [1934, 1937], who found that the cervical muscle, examined *in vitro*, was insensitive to the oxytocic principle of the posterior lobe of the pituitary and also that there was evidence of reciprocal activity between the cervix and the horn of the uterus. Observations on the activity of the horn of the uterus in different species indicate that there may be wide discrepancies between the behaviour of excised tissues and the behaviour of the same tissues in the intact animal. In view of this difficulty and its importance in relation to the theory of parturition we thought it advisable to repeat the experiments on the cervix *in vivo*. By investigating three species, guinea-pig, rabbit and cat, we hoped to make our conclusions as general as possible, since species differences are so common in this field.

METHOD

The movements of the cervix were recorded by placing a small rubber balloon in the cervical canal. These movements are very small, and it was soon apparent that the usual methods of volume recording by tambours were too easily upset by environmental changes at the sensitivity required. A new optical device was therefore designed and has already been described [Adler, Bell & Knox, 1941]. This apparatus has the advantage of giving immediate records on smoked paper. In all cases the capacity of the balloon was 0·3 c.c., this being approximately the capacity of the prism. The balloon was tied on the end of a fine metal cannula which carried a moveable sleeve with a cup-shaped end. After separating the bladder and vagina by blunt dissection the undistended balloon was inserted through a mid-line incision in the vaginal wall into the cervical canal, and the cup-shaped end was stitched to the lip of the cervix (Fig. 1). The cannula was then pulled back through the sleeve until the balloon was just touching the cup. The balloon was now fixed in the cervical canal, and could not slip forward into the uterine horn. The balloon and cannula were filled and connected to the recording device by a waterfilled tube; the balloon was thus under a pressure of 7 cm. of water. At the end of each experiment the position of the balloon was checked by inspection or by fixing the uterus *in situ* with formalin without disturbing the balloon. When the uterus was laid open the cervix was easily recognized because of the granular character of its lining membrane. The apparatus was calibrated at the end of each experiment by substituting a graduated pipette for the cervical balloon. A scale was drawn on the smoked paper corresponding to hundredths of a c.c. change in volume.

The cornual movements were recorded by the cannula described by Bell & Robson [1936] and records were made directly on the smoked drum; no photoelectric amplification was necessary. We are satisfied that contractions of the cornu did not affect the cervical record. The experimental evidence for this is given later under the heading of 'evidence for the separate identity of the cervix and cornu'.

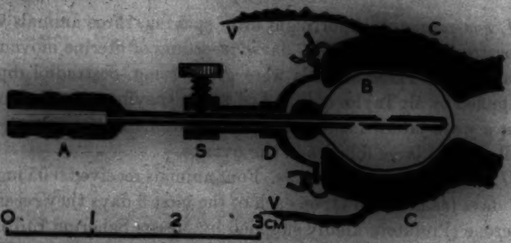

Fig. 1. Diagram of cannula and balloon in position in the cervical canal. *C*, cervix. *V*, vagina. *A*, cannula. *B*, balloon. *S*, sleeve. *D*, cup-shaped end of sleeve.

A small glass cannula with a rubber stopper was tied into the external jugular vein. All drugs were injected through this stopper by means of a hypodermic needle. The rigidity of the stopper prevented any alteration of volume on withdrawal of the needle, no blood was sucked into the cannula and clotting was avoided.

In certain of the experiments it was necessary to compare the effect of a quick injection with a continuous injection. A quick injection means that it was given in the usual way by means of a hypodermic syringe, the total time of injection being only a few seconds. A continuous injection, on the other hand, lasted in these experiments about 20 min. A block of wood on a lead screw driven by an electric motor through reduction gearing gradually pressed out the contents of a 10 c.c. syringe which was connected to the vein by a narrow rubber tube ending in a needle passed into the stopper of the non-clotting cannula.

Details of the pre-operative treatment of the animals will be found in the description of the results. Guinea-pigs were anaesthetized with chloralose given subcutaneously (7 mg./kg.), supplemented by ether when necessary. Rabbits and cats were anaesthetized with nembutal (50 mg./kg.) given subcutaneously. In all experiments an attempt was made to estimate the threshold dose of oxytocin for both cornu and cervix, using a specially purified oxytocic hormone (purified pitocin), kindly supplied by Dr White of Parke Davis and Co. The other drugs used were adrenaline chloride (Parke Davis and Co.), acetylcholine (Pragmoline, May and Baker Ltd.), and vasopressin (Pitressin, stock sample, Parke Davis and Co.).

RESULTS

The results of the experiments are summarized in Table 1. In the following description the word 'uterus' should be taken to include both cornu and cervix.

Guinea-pigs

Group I, non-pregnant. A fortnight after spaying, three animals were given 0·05 mg. oestrone in oil daily for 7 days; recording of uterine movements took place 3 days later. Three animals were given 1 mg. oestradiol dipropionate (kindly supplied by Mr Taylor of Ciba Ltd.) after spaying, the experiment being carried out a week later. Lastly, three intact animals were operated upon at oestrus; all showed large follicles in the ovaries.

Group II, non-pregnant guinea-pigs. Four animals received 0·05 mg. oestrone in oil for 7 days (as in group I); on each of the next 3 days they received 5 mg. of progesterone (Proluton, kindly supplied by Messrs Schering Ltd.). Records were made on the day after the last injection of progesterone. In addition, two intact animals were operated upon 7 days after the opening of the vagina (which occurs at oestrus), large corpora lutea being present.

Group III, pregnant guinea-pigs near term. These five animals were from 55 to 60 days pregnant. The age of the pregnancy was judged from the length of the foetuses [Bell, 1941].

Group IV, parturient guinea-pigs. Except in one case, these seven animals were operated on within 12 hr. of parturition. In one instance the experiment began within half an hour of delivery.

The spontaneous movements were in all cases measured at the beginning of the experiment before any drugs, except the anaesthetic, had been introduced into the circulation. The cervix showed spontaneous movements in twenty out of twenty-seven experiments; the amplitude depended more on the size of the cervix than on the hormonal condition of the animal. In those cases in which no cervical activity was noted early in the experiment it became evident later on. The frequency of the spontaneous contractions was higher in the cervix than in the cornu. As has been previously shown [Bell & Robson, 1936; Adler & Bell, 1943] there is very little difference between the behaviour of the cornual muscle in groups I and II; the threshold dose of pitocin in group III is much lower [Bell, 1941]. The cervix responded to injections of pitocin by contracting in all cases except two, in which the dosage may have been in-

Table 1. Summary of results

Group	No. in group	Average wt. g.	Extent and period of spontaneous movements		Threshold dose of pitocin in o.u.		Effect of adrenaline		Effect of acetylcholine		Effect of pitressin
			Cornu	Cervix	Cornu	Cervix	Cornu	Cervix	Cornu	Cervix	
I. Guinea-pigs treated with oestrin or those with ovarian follicles	9	473	2·8 cm. 6 min. 20 sec.	0·002 c.c. 2 min. 10 sec.	0·011 C (8) 0·1 N (1)	0·034 C (6) N (3)	N (5) R (3)	C (3) N (3) R (2)	N (3) C (1)	N (4)	—
II. Guinea-pigs treated with oestrin and progestin, or those with corpora lutea	6	427	2·4 cm. 1 min. 40 sec.	0·002 c.c. 1 min.	0·03 C (6)	0·06 C (2) 0·1 N (2) 0·005 N (2)	R (3) N (3)	N (4) C (1) R (1)	N (4) I (1)	N (5)	—
III. Pregnant guinea-pigs near term	5	588	1·7 cm. 5 min. 30 sec.	0·036 c.c. 6 min.	0·003 C (4)	0·011 C (4)	N (1) R (2) C (1)	N (2) R (1) C-R (1)	N (1)	N (1)	N (2)
IV. Parturient guinea-pigs	7	545	1·6 cm. 4 min. 40 sec.	0·019 c.c. 2 min. 40 sec.	0·018 C (4) 0·02 N (2)	0·015 C (6)	R (4) N (1)	C (2) I (1) R (1) N (1)	N (1)	N (1)	C of both (1)
V. Non-pregnant rabbits treated with oestradiol	3	2500	1·7 cm. 50 sec.	0·03 c.c. 2 min.	0·07 C (3)	1·0 C (1) 0·25 C (1) 0·5 R (1)	C (3)	C (1) C-R (2)	C (1)	C (1)	C (2) cornu N (2) cervix
VI. Pregnant rabbit	1	2600	1·5 cm. 1 min.	0·01 c.c. 1 min.	0·05 C (1)	0·05 N (1)	—	—	—	—	—
VII. Parturient rabbits	5	2250	3·8 cm. 1 min. 20 sec.	0·04 c.c. 40 sec.	0·017 C (5)	0·04 C (5)	C (2) C-R (1) I (1) R (1)	C-R (3) C (2)	C (2)	R (1) N (1)	C (2) cornu C (1) cervix N (1) cervix
VIII. Pregnant cat	1	7200	2·0 cm. 5 min.	0·01 c.c. 5 min.	0·1 C (1)	0·1 C (1)	R (1)	R (1)	—	—	—
IX. Parturient cats	2	2600	Nil	0·01 c.c. 30 sec.	0·02 C (2)	0·01 C (1) 0·3 C (1)	R (2)	C (1) C-R (1)	N (1)	N (1)	—

Abbreviations: C = contraction, R = relaxation, N = no effect, I = inhibition, C-R = contraction followed by relaxation. Numbers in brackets refer to number of cases, e.g. C (3) means a contraction obtained in three animals. o.u. = oxytocic units.

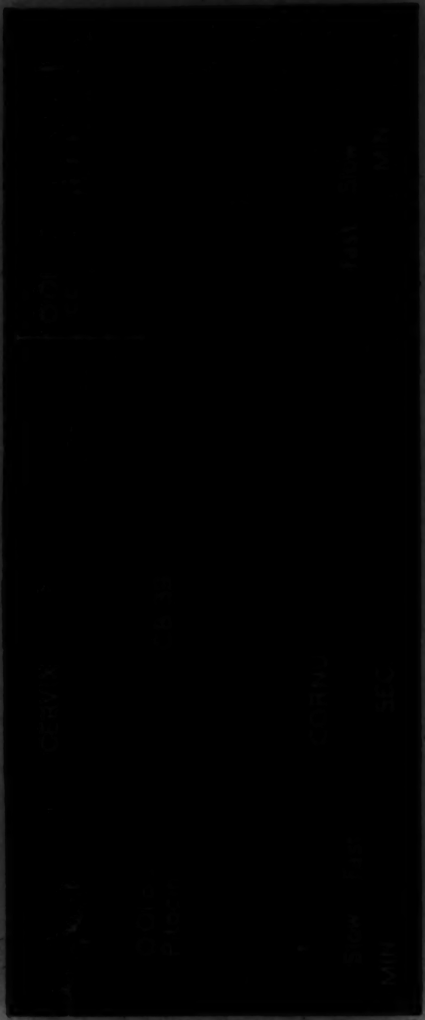

Fig. 2. CB 39. Guinea-pig, non-pregnant. Spayed and given 1 mg. oestradiol dipropionate. Tracing made one week later. Contraction of cornu and cervix after intravenous injection of 0·01 c.u. pitocin. Calibration scale in hundredths of a c.c. has been superimposed on the cervical tracing. In this and the subsequent tracings contraction is indicated by an upstroke.

adequate. The threshold dose required to produce a contraction of the cervix was about three or four times that required to produce a contraction of the cornu in the same animal. In addition, the cervical response to a given dose of pitocin was of very much shorter duration than that of the cornu; for example, in the case of CB 39 (Fig. 2), after the intravenous injection of 0·01 o.u. (oxytocic units) of pitocin, the rise of tone lasted for 3 min. in the case of the cervix, and for 20 min. in the case of the cornu. To explore this question further, continuous injections were given. Thus CB 53 was given 0·13 o.u. pitocin in 18 min.; the cornu showed increased activity for 1½ hr., whereas the effect on the cervix was over in about 18 min.; thereafter for about ¾ hr. the cervix was less active than before the injection (Fig. 3). A characteristic feature of many of the tracings, shown in both Figs. 2 and 3, was that after the short period of tonic contraction of the cervix following injection of pitocin there was for a time increased clonic activity, i.e. isolated contractions with intervening periods of complete relaxation. As shown in Fig. 3, while the cornu was still tonically contracted under the influence of pitocin, the intervals between the clonic contractions of the cervix steadily lengthened and were for a time much greater than before the injection of pitocin.

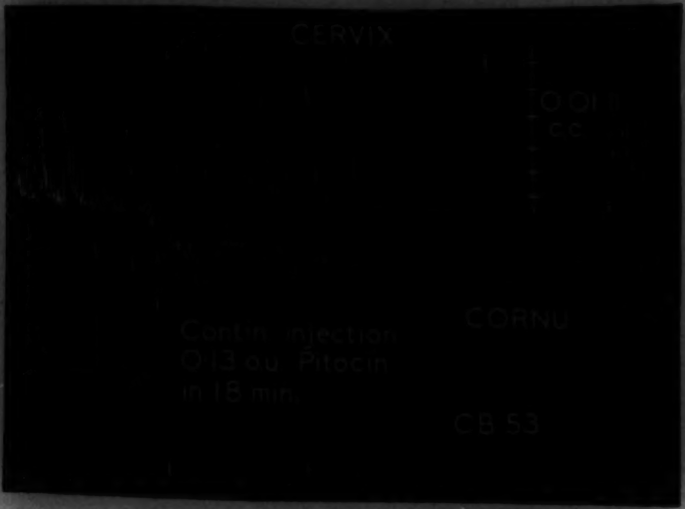

Fig. 3. CB 53. Guinea-pig, 5 hr. post-partum. Continuous injection of pitocin. Time tracing in minutes.

Rabbits

Group V, non-pregnant rabbits. These three animals were spayed and 5 days later were given 1 or 2 mg. oestradiol dipropionate; 1 week later the movements of the uterus were recorded.

Group VI, pregnant rabbit. From the length and weight of the foetuses [see table in Needham, 1931] this animal was approximately 27 days pregnant.

Group VII, parturient rabbits. These five animals were operated upon within 12 hr. of full-time parturition. In one case, one living foetus still remained within the uterine horn, two having been born shortly before the experiment.

The cervix and the cornu showed spontaneous movements in all nine cases, the largest amplitudes being shown by the parturient animals. The threshold dose of pitocin for the cervix was in all cases higher than that for the cornu, the range being from three to ten times. There was very little difference between the behaviour of the uteri in the three groups, except that the parturient animals were more reactive to oxytocin than the non-pregnant animals, in spite of the very large dose of oestrogenic material given to the latter.

Cats

Three non-pregnant cats were spayed and injected with large doses of oestrin, up to a total of 5 mg. of oestradiol dipropionate at intervals over 1 month. In all cases the cervix remained extremely narrow and rigid and did not permit the passage of the balloon.

Group VIII, pregnant cat. This animal was approximately 1 week prepartum judged by the length of the foetuses [see data in Windle & Griffin, 1931].

Group IX, parturient cats. Records were obtained from these two animals within 12 hr. of normal full-time delivery.

In this species it was again found that the cervix contracted after an injection of pitocin, was less reactive than the horn, and that the effect on it of a given dose was shorter.

Action of adrenaline

In guinea-pigs this drug, administered intravenously, usually produced a relaxation of the cornu, but was often without effect in the dosage used (usually not more than 50μg.); this is in agreement with Gunn & Gunn [1914]. Its effect on the cervix was extremely variable, contractions and relaxations being produced in approximately equal numbers. Newton found that the guinea-pig cervix *in vitro* was contracted by adrenaline. There were only five cases of reciprocal activity between the cervix and the cornu (Fig. 4). In the rabbit, on the other hand, the cervix always contracted after injection of adrenaline, although in some cases this was followed by a relaxation (Fig. 5); the effect on the cornu was usually a contraction [see also Langley, 1901; Dale,

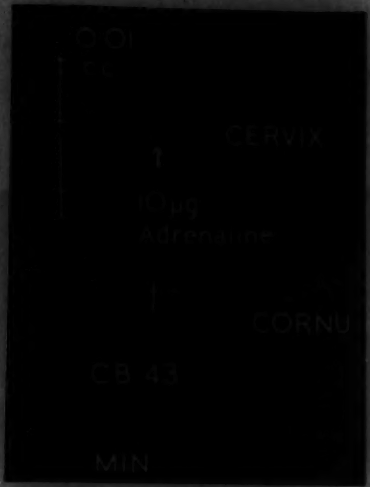

Fig. 4. CB 43. Guinea-pig at oestrus. Effect of intravenous injection of 10 µg. adrenaline. The steady fall in the cervical tracing is due to a very slow leak in the recording system.

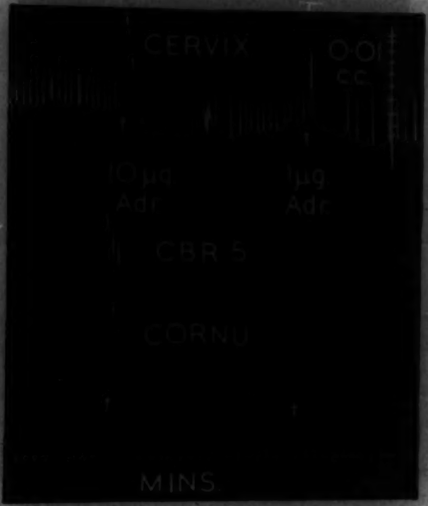

Fig. 5. CBR 5. Rabbit, 11 hr. post-partum. Effect of intravenous injection of 10 µg. adrenaline.

1906]. There was thus no clear-cut instance of reciprocal activity in this species. The parturient cats both showed reciprocal activity to adrenaline, the horn relaxing [see also Dale, 1906] while the cervix contracted (Fig. 6). In a pregnant cat adrenaline relaxed both parts of the uterus; Dale [1906] and Cushny [1906] obtained a contraction of the horn. Kehrer [1907] found that the pregnant cat cervix was contracted *in vitro* by adrenaline. In our three spayed cats treated with oestradiol, adrenaline caused a relaxation of the cornu; this is in agreement with the findings of Dale and Cushny in virgin cats.

Fig. 6. PC 1. Cat. 7 hr. post-partum. Effect of injection of 150μg. adrenaline intravenously. Time in min.

Action of acetylcholine

Acetylcholine usually had no effect on the activity of the cervix or cornu. It ought to be stated, however, that this drug was administered towards the end of the experiments when the effects of pitocin and adrenaline had been investigated, and accordingly not much stress ought to be laid on the absence of effect.

Action of pitressin

The action of pitressin could be fully accounted for on the basis of its oxytocin content.

DISCUSSION

There is no doubt now that the cervix uteri can be made to contract in the living animal under the influence of the oxytocic principle of the posterior lobe of the pituitary gland. This is in direct contrast to the results obtained by Newton [1934, 1937] who found that the cervix in the goat, rat and guinea-pig when examined *in vitro* was quite insensitive to oxytocin. The large molecule of oxytocin may penetrate the muscle cells of the cervix *in vitro* with difficulty, but it may obtain access more easily *via* the circulation. The small molecule of adrenaline can apparently reach the cervical muscle cells readily *in vitro*. The uterine muscle of the monkey [Bell, 1942] behaves similarly, being more reactive to oxytocin *in vivo* than *in vitro*; also *in vitro* it is easily stimulated by the small molecule of histamine. This is, however, not an invariable finding, the uterine muscle of the guinea-pig, for example, being more sensitive to oxytocin *in vitro* than *in vivo* [Bell & Robson, 1936; Bell, 1941].

On general principles it would seem wiser to build a theory of parturition on evidence obtained from experiments performed *in vivo*. The question then arises as to the position of the oxytocic principle of the posterior lobe as *the* oxytocic agent concerned in parturition. Newton says [1934]: 'If labour is due to the action of an oxytocic substance the advantages of insensitivity to such bodies on the part of the circular cervical muscle are obvious.' Again [1937]: 'The most severe, and at the same time the most easily demonstrable, type of inco-ordination would be a simultaneous contraction of the cervix and cornu of the uterus, and in the author's opinion this would, if brought about by the oxytocic principle, definitely settle all doubts on the score of its physiological activity.' The present work at first sight seems, therefore, to require that the oxytocin theory of parturition be discarded. There are, however, two mitigating features: first, the cervical threshold is higher than the cornual, and secondly, the effect of oxytocin on the cervix is relatively transient.

If we accept the possibility of a certain balance of strength between the cervical and cornual musculature we may fit the present findings into an oxytocic theory of parturition in the following way. In the latter part of pregnancy the uterine movements are of considerable amplitude; it may be that they are spontaneous or it may be that they are caused or augmented by oxytocin. The cervix may during this time remain unaffected. When the secretion of oxytocin increases and the first stage of labour begins the cervix will be stimulated and the constriction of the cervix, while the body is also showing active contractions, will aid in the development of the lower uterine segment, and perhaps also in the moulding of the foetal head. After a time, however, the cervix will cease to be affected by the oxytocic principle and dilatation of the uterine os can then take place. In addition, the peculiar behaviour of the cervical musculature towards the oxytocic hormone may have the effect of preventing precipitate labour, and may be a good example of co-ordination rather than a severe type of inco-ordination as Newton would have it.

Cervical dystocia. It may be mentioned that true cervical dystocia is, apart from trauma or disease, an infrequent occurrence [Sackett, 1941], being usually encountered in elderly primigravidae with a history of constitutional deficiency and genital underdevelopment. If, as suggested above, the cervix plays something more than a mere mechanical role in parturition this association of cervical dystocia with symptoms of endocrine disturbance may be significant.

Preparation of the cervix for parturition. The cervix in the pregnant and parturient animals was always larger than in the non-pregnant animals, and was in addition somewhat oedematous. This hypertrophy may be due to the same causes, hormonal and mechanical, as those which stimulate the growth of the pregnant uterus. The guinea-pig results indicate that the increase in the size of the cervix, estimated directly and from the extent of the spontaneous movements, cannot be accounted for on the basis of the action of either or both of the ovarian hormones. The reactions of the pregnant and non-pregnant cervix to drugs show no clear-cut differences except perhaps that the threshold to pitocin is lower in the case of the pregnant guinea-pigs. In the case of the pregnant rabbits the cervix appeared to be more oedematous but was very little larger than in the spayed animals treated with large doses of oestradiol. The cervix of the parturient rabbits was much more reactive to oxytocin than that of the spayed animals, although the latter had been given very large doses of oestradiol; this statement applies also to the horn in these groups (V and VII). These findings make it doubtful if oestrogenic stimulation is the complete explanation of the high reactivity observed at parturition. This is in conflict with the usual description of the cause of the high reactivity at parturition in rabbits [Robson, 1933] which was founded on experiments *in vitro*. The cervix in groups V and VII was highly reactive to adrenaline (in some cases less than 1 μg. was effective), but there was no distinction between the two groups. In the case of the cat there is no doubt whatever that intensive oestrin treatment by itself cannot account for the very great change from the small, very firm cervix of the non-pregnant condition to the large and oedematous cervix of parturition. The number of animals in groups VIII and IX is too small to allow any conclusions to be drawn from the actions of pitocin and adrenaline.

Reciprocal activity. Reciprocal activity between the cervix and cornu is not prominent in the present work. *In vitro*, the cervix of the goat and guinea-pig is contracted by adrenaline, while that of the rat is not affected, and it is only in the goat that a clear-cut case for reciprocal activity has been made out; in the guinea-pig reciprocal activity is probably present during di-oestrus and early pregnancy, but not at oestrus [see Newton, 1934, 1937]. All the evidence from the three species we have investigated shows that reciprocal activity to adrenaline is by no means general, and the variability of the results makes it difficult to find any role for this substance in pregnancy or parturition.

Evidence for the separate identity of the cervix and cornu. Newton's experiments *in vitro* demonstrate that pharmacologically the cervix and cornu are quite different tissues. Our evidence obtained *in vivo* strongly suggests that these two parts of the uterus are also physiologically separate. *In vivo* the spontaneous activity of the body and the cervix of the uterus was not very dissimilar, as will be seen from the figures, but there were differences in detail in the contractions (e.g. fast portion of Fig. 2). Also cervical contractions were observed in the absence of cornual contractions and vice versa. Usually, however, when the cornu contracted the cervix contracted a few seconds later (fast portion of Fig. 2). The following series of stop-watch observations on successive spontaneous movements in a parturient rabbit (CBR 5) shows that there may be considerable variations in the interval between cornual and cervical contractions (+ means that the cervix is leading, and − means that the cornu is leading by the stated number of seconds): +2, +4, +2, −2, −7, −2, −6, −9, −11, −11, −11, −10, −7, −7, −3, −2, −6, −5, −6, −5, −11, −1, 0, 0, +2, +2, +3, 0, −7, −5, 0, −9, −10, −7, −16, −11, −18, −19. Moir [1934] found that in the puerperal human subject the cervical rhythm appeared to be independent of that of the fundus, but that there were a few tracings in which the two were plainly co-ordinated. When in addition to these observations we take into account the examples of reciprocal activity to adrenaline, and the differences in the responses of the cervix and cornu to pitocin, we must conclude that the cervix is not merely the end of the uterus but that it is a specially modified part of that organ with a physiological and pharmacological identity.

Summary

1. The movements of the smooth muscle of the cervix and cornu of the guinea-pig, rabbit and cat have been examined in the living animal in the non-pregnant, pregnant and parturient states. In the majority of cases the cervix showed spontaneous movements of small amplitude, but of greater frequency than those of the cornu.

2. The cervix in nearly all cases contracted after intravenous injection of purified oxytocin; the threshold dose was usually several times higher than that for the cornu; but the cervical response to a given dose of oxytocin was of much shorter duration than that of the cornu. These results are very different from those of Newton [1934, 1937] who found that the cervix *in vitro* was insensitive to oxytocin.

3. The action of adrenaline on the guinea-pig cervix was very variable. In the rabbit and in the parturient cats the cervix contracted; in a pregnant cat adrenaline relaxed the cervix.

4. The present findings may be readily fitted into an oxytocic theory of parturition. It is difficult to account for the condition of the cervix at parturition on the basis of oestrogenic stimulation. The cervix appears to be a specially modified part of the uterus with a physiological and pharmacological identity.

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THE EXCRETION OF HISTAMINE IN URINE

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Attempts to demonstrate the presence of histamine in normal urine by biological methods have been, so far, unsuccessful. Even after injection of large amounts of histamine none could be detected in the urine [Dale & Laidlaw, 1910; Oehme, 1913; Guggenheim & Loeffler, 1916]. On the other hand, Best & McHenry [1930] report that neutralized HCl hydrolysates of dog's urine lower the blood pressure of the atropinized cat and suggest without, however, any further evidence, that the effect might be due to histamine. On this assumption they estimated the histamine equivalent of the two urine samples which they analysed as 0·2μg./c.c. Macgregor & Peat [1933] detected histamine in the urine when large amounts of the substance were added to the blood in a perfused kidney. The concentration in the blood in these experiments must have been much higher than ever occurs in the intact animal. Ungar & Pocoulé [1937] found no histamine in human urine. On the available evidence Feldberg & Schilf [1930] and Gaddum [1936] conclude that histamine is probably not normally excreted by the kidneys.

The results of isolation of histamine by chemical methods from large amounts of urine were more satisfactory. According to Koch [1913] histamine can be isolated from the urine of parathyroidectomized dogs. Revoltella [1927] claims that histamine is excreted in eclampsia, and Kapeller-Adler [1941] isolated from the urine of a woman suffering from severe pre-eclamptic toxaemia about 1 mg. of histamine base per litre of urine. Ackermann & Fuchs [1939] obtained 0·9 mg. of histamine dipicrate per litre of normal urine; the amount was too small for unequivocal identification.

In the course of our experiments we found that extracts of normal urine prepared by a modified technique of Barsoum & Gaddum [1935] are inactive when tested for histamine unless they have been previously hydrolysed in acid. After hydrolysis in HCl the extracts show unmistakable evidence of containing histamine which greatly varies in amount in different specimens of urine. It will be shown that histamine is normally excreted in a conjugated and inactive form from which the active base can be released by hydrolysis.

CONJUGATED HISTAMINE

Conjugated histamine like the free base is readily adsorbed from the urine by charcoal (B.D.H. decolorizing charcoal) from which it can be released by repeated washing with acidulated alcohol. A large amount of conjugated histamine can be thus collected on a relatively small amount of the adsorbent. The eluate is almost free from inorganic salts and from a considerable amount of the organic substances of the urine. The following method is used by us for the preparation of conjugated histamine.

Method. 10 c.c. urine are shaken with about 0·25 g. charcoal and filtered under suction. The filtrate is preserved for further analysis and the charcoal on the filter is washed with cold water. After allowing most of the water to drain, 10 c.c. 0·3*N* HCl in 95% alcohol are passed through the charcoal. The first portion of acidulated alcohol removes the remaining water. Further portions of acidulated alcohol, 10 c.c. each, are then passed through the filter. Each portion is passed 3–4 times, about 40–60 c.c. being used in all. Beginning with the second portion the strength of the acidulated alcohol may be reduced to 75%. All the alcoholic eluates, including the first, are joined together and carefully neutralized. The charcoal filtrate and the alcoholic eluate are divided into two parts. One part of each is hydrolysed in HCl while the respective remainders are dried *in vacuo* on a boiling water-bath. For the hydrolysis 10 c.c. conc. HCl and about 50 c.c. water are added and the mixture is boiled for 1·5 hr., water being occasionally added to prevent desiccation. Towards the end of the hydrolysis the volume of the mixture is reduced to a few c.c. The two hydrolysates are evaporated to dryness on a water-bath under suction. Four portions of 10 c.c. 95% alcohol are added and distilled off in order to remove the HCl. The dried residues of the hydrolysed and non-hydrolysed fractions of the filtrate and of the eluate are then extracted by a technique similar to that of Barsoum & Gaddum [1935]. The extraction is made with four lots, 3 c.c. each, of absolute alcohol saturated with NaCl. The residue usually forms a crust at the bottom and on the sides of the flask, so that a certain amount of scraping with a glass rod is necessary during the extraction. The alcoholic extracts are filtered and evaporated to dryness. The dry residue is taken up in 4·4 c.c. water and neutralized to bromo-thymol blue with x c.c. *N* NaOH; $(0·6-x)$ c.c. *N* NaCl (5·85%) are then added to make the total volume equal to 5 c.c. When more than 0·6 c.c. of the normal alkali is required the neutralization is completed with *N*/7 NaOH after which Tyrode solution is added to any desired volume.

As a result of this procedure we have now four extracts: a hydrolysed and a non-hydrolysed extract of the filtrate and a hydrolysed and a non-hydrolysed extract of the eluate. At this stage of preparation the four extracts are not suitable for quantitative tests, since they all contain a variable amount of relaxing material which is especially noticeable when the assay is made on the rectal caecum of the fowl. The relaxing material can be removed by adsorption on aluminium oxide (B.D.H. 'for adsorption'). Each extract is treated with about 1 g. of the adsorbent and filtered. The *p*H of some extracts has to be readjusted after treatment with aluminium oxide by addition of a trace of HCl. Adsorption on Al_2O_3 should not be omitted unless the histamine equivalent of an extract is so high that it can be diluted 5–10 times. The four extracts are now ready for the assay. As a routine the tests were made on the guinea-pig's ileum and on the rectal caecum of the fowl. The test object was suspended in a slightly atropinized (1 in 10^7) Tyrode solution in a bath of 5 c.c. capacity at 36° C. In other details we followed the usual procedure as recommended by Barsoum & Gaddum [1935].

The charcoal filtrate. There is no significant difference between the hydrolysed and non-hydrolysed fractions of the filtrate. After adsorption on Al_2O_3 both evoke contractions of the ileum, which are, however, not typical of histamine. The contractions are not sustained; on repetition of the injection they diminish in strength, but large doses of the extract may cause a temporary diminution of the sensitivity of the ileum to histamine, and the contractions are not abolished by the selective paralysis of the ileum with large doses of histamine [Barsoum & Gaddum, 1935] or with traces of the drug F 933 [Ungar, Parrot & Bovet, 1937]. Expressed in terms of histamine acid phosphate the activity of these extracts varies for human urine between 0·03 and 0·12 μg./c.c. After five or six injections the ileum becomes insensitive to these extracts, still retaining its sensitivity to histamine. Although extracts of the filtrate contain no histamine their occasional assay is useful as providing a check on the completeness with which the histamine has been adsorbed on the charcoal.

The charcoal eluate. The unhydrolysed extracts of most charcoal eluates of dog's and human urine are inactive when tested on the ileum and cause a conspicuous relaxation of the rectal caecum. The relaxation is abolished by treatment of the extracts with Al_2O_3. The histamine equivalent of those few extracts which evoke a contraction of the ileum is below 0·1 μg./c.c. Injection of non-hydrolysed extracts into an atropinized dog, in amounts equivalent to 40 c.c. urine, has no effect on the blood pressure. However, under certain experimental conditions and in some animals in which histamine is normally excreted in a free form or when some histamine has been added to the urine, the assay of the non-hydrolysed extracts gives positive results. As will be shown below, the non-hydrolysed extracts of the eluate can be used for quantitative estimation of free histamine in the urine. After acid hydrolysis the extract of the eluate shows a striking increase of activity. The extent of this effect can be seen from Table 1 and Fig. 1.

TABLE 1. Showing the activity of the charcoal eluate before and after hydrolysis in HCl. The examples given here are taken at random from experiments made under different conditions. The assays are made on the guinea-pig's ileum and the histamine equivalents are given in μg./c.c. histamine acid phosphate. Before testing, the extracts were treated with Al_2O_3.

Human urine		Dog's urine	
Before hydrolysis	After hydrolysis	Before hydrolysis	After hydrolysis
Trace	0·60	Trace	7·20
Trace	0·55	Trace	0·25
Trace	2·20	Trace	0·15
0·06	0·09	0·03	4·10
<0·06	0·30	Trace	10·50
<0·06	5·20	<0·05	54·00
Trace	1·90	0·06	70·00

Fig. 1. Guinea-pig's ileum; dog's urine. Effect of acid hydrolysis of urine extracts prepared by the charcoal method. *E* 1, non-hydrolysed and *E* 2, hydrolysed extracts; doses in c.c. of urine. *H*, standard solution of histamine acid phosphate; doses in μg. All the extracts were adsorbed on Al_2O_3. The histamine equivalent of the urine was estimated as 5·5 μg./c.c.

The increase in the histamine-like activity of urine extracts after hydrolysis can be due either to the destruction of some antagonizing substances interfering with the contraction of the ileum or to a release of the active principle from a conjugated and inactive form. The first possibility is excluded by the fact that histamine when added, even in traces, to the non-hydrolysed extracts can be quantitatively estimated on any of the usual test objects. No interfering substances can therefore be present. As a result of these observations we conclude that the histamine-like substance of urine is normally excreted in a conjugated inactive form. Table 1 shows that the concentration of this substance in the urine may vary to a remarkable extent. The study of its excretion in different species of animals and under different experimental conditions is of obvious importance.

Precautions necessary during the preparation and hydrolysis of conjugated histamine. The amount of charcoal used for the adsorption of conjugated histamine should not be reduced below 0·2 g. per 10 c.c. urine if quantitative results are desired. When half this amount is used some of the substance escapes adsorption. When large amounts of urine are employed at least 150–200 g. charcoal per l. urine should be used. The necessary amount will vary with the brand of charcoal. Variations in the *p*H of urine between 5·5 and 9·0 do not affect the completeness of the adsorption.

The recovery of conjugated histamine from the charcoal with non-acidulated absolute alcohol is incomplete. The acidity of the alcohol should not be below 0·15 *N* HCl and the alcohol not below 70%. After many trials we found the recommendations given earlier to answer the purpose, namely 0·3 *N* HCl in 95% alcohol for the first and in 75% alcohol for the subsequent washings. When large amounts of charcoal are worked on, it is advisable to remove it from the filter, mix with about 300–400 c.c. acidulated alcohol and refilter. The process must be repeated several times.

The duration of the hydrolysis should not be less than 1 hr. When shortened to 30 or 45 min. the yield of free histamine is about 75 and 90% respectively. We always continue the hydrolysis for 1·5 hr., but further prolongation is without harm. Conjugated histamine, like the free base, is soluble in alcohol and water and insoluble in ether.

Histamine can be released from the conjugated form also by hydrolysis in alkali. However, this method presents the disadvantage that the released histamine itself undergoes a gradual destruction. For example, hydrolysis of a 5 c.c. sample of urine in acid yielded 2·25 μg. histamine. Hydrolysis of a second sample in 0·2 *N* NaOH released 0·85 μg., and a further hydrolysis of the same sample in acid yielded 0·8 μg., giving a total of 1·65 μg. The difference of 0·6 μg. (2·25–1·65) was presumably due to the destruction of histamine by the alkali. A similar alkali hydrolysis of 2·25 μg. of released histamine reduced it to 0·7 μg.

Non-hydrolysed eluates of urine can be kept for a long time; one sample remained in the laboratory for over 3 years without much deterioration. The dried residue of the eluate has a waxy appearance and obviously contains a large amount of impurities. In view of the limited facilities at our disposal we have not attempted to purify the extract. At present any speculation as regards the composition of conjugated histamine must be left over until this derivative is obtained in a pure form.

Evidence that the active substance is histamine. The typical contractions of the ileum and of the rectal caecum evoked by the hydrolysed extracts are abolished by selective paralysis with large doses of histamine or traces of piperidinomethylbenzodioxan—F 933 (Fig. 2). The extracts vigorously contract the guinea-pig's bronchi and lower the blood pressure of the atropinized cat (Fig. 3). Assays performed on the ileum, rectal caecum and the blood pressure show good agreement. On intradermal administration in man the extracts evoke a conspicuous triple response with formation of blisters. Non-hydrolysed extracts are in all these respects completely inactive.

Fig. 2. Effect of selective paralysis of the guinea-pig's ileum with F 933 on its response to histamine (*H*) and to the hydrolysed extract of dog's urine (*E*). Doses in c.c. urine and μg. histamine acid phosphate. F 933 was administered after the second contraction of the ileum. The ileum continued to respond normally to KCl and alkali.

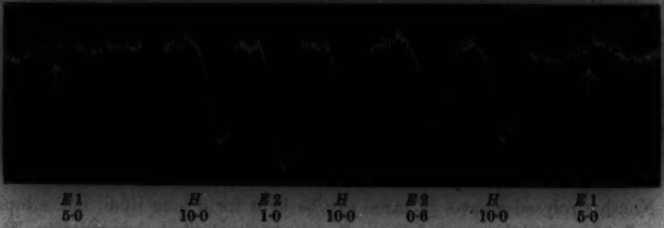

Fig. 3. Effect of urine extracts on the blood pressure of the atropinized cat. *E* 1, non-hydrolysed extracts of dog's urine; *E* 2, hydrolysed extracts of the same urine; *H*, histamine. Doses in c.c. urine and μg. histamine acid phosphate.

Attempts to demonstrate the action of histaminase on urine extracts were at first unsuccessful. It was found that the enzyme is entirely inactive in the presence of urine, and that this inhibitory influence is retained by the extracts of the urine even after acid hydrolysis. On diluting the urine or the extracts with water the enzyme becomes more active. With a tenfold dilution the inhibition of the enzyme is negligible. Taking this into account we tested the action of histaminase on the hydrolysed and non-hydrolysed extracts after different degrees of dilution with water (Table 2).

TABLE 2. Action of histaminase prepared by the method of Anrep, Barsoum & Talaat [1936]. (A), on histamine acid phosphate added to human urine (sp. gr. 1·025); (B), on histamine acid phosphate added to Tyrode solution; (C), on the non-hydrolysed urine extract (conjugated histamine); (D), on the hydrolysed extract of the same urine (released histamine). The samples were incubated at 37° C. for 30 min. The same amount of enzyme solution was added to each sample. The amount of histamine added to the total volume of each sample A and B and the histamine equivalent of each sample C and D before incubation was 30 μg. The histamine equivalent of samples C was determined after acid hydrolysis. The dilution of the samples is shown in the first column. The amount of histamine in the total volume of each sample after incubation is given in the table in μg. of histamine acid phosphate.

Dilution of sample	A Histamine in urine	B Histamine in Tyrode solution	C Conjugated histamine	D Released histamine
0	30·00	30·00	30·00	30·00
4	18·00	0·50	30·00	16·00
8	6·00	0·70	30·00	4·50
16	0·75	0·30	30·00	0·50
32	0·50	0·50	30·00	0·60

It is evident from Table 2 that histaminase does not act on conjugated histamine whatever the dilution (C), while the product of its hydrolysis is destroyed at the same rate (D) as histamine which has been added to urine (A). All these tests support the conclusion that the active principle of hydrolysed urine extracts is histamine.

QUANTITATIVE DETERMINATION OF TOTAL CONJUGATED AND FREE HISTAMINE

Total histamine. Adsorption on charcoal is not essential for quantitative estimation of the total histamine in urine. Satisfactory results can be obtained by directly hydrolysing 0·1–5·0 c.c. urine in HCl and then extracting the active principle by the modified technique of Barsoum & Gaddum. For this purpose the urine is treated in the same way as described for the charcoal eluate. Before the assay the final extracts must be adsorbed on Al_2O_3. The importance of this step is made clear by Table 3 and Figs. 4 and 5, which

TABLE 3. Showing the effect of adsorption of extracts of human urine with aluminium oxide. The assay of each extract was made on the guinea-pig's ileum and on the rectal caecum of the fowl. The histamine equivalents are given in μg./c.c.

	Guinea-pig's ileum		Fowl's rectal caecum	
	Before adsorption	After adsorption	Before adsorption	After adsorption
1	2·2	2·2	Relaxation	2·0
2	0·6	0·9	Relaxation	0·8
3	0·7	0·8	Relaxation	0·8
4	0·9	1·3	Relaxation	1·1
5	0·2	0·5	Relaxation	0·6
6	<0·05	0·12	Relaxation	No relaxation
7	0·25	0·3	Relaxation	0·25
8	Relaxation	0·07	Relaxation	No relaxation

Fig. 4. Guinea-pig's ileum. Effect of adsorption of an extract of human urine with Al_2O_3. The urine extract was prepared by the method of direct hydrolysis. *E* 1, non-hydrolysed extract of urine. *E* 2, non-hydrolysed extract of urine adsorbed on Al_2O_3. *E* 3, hydrolysed extract of urine. *E* 4, hydrolysed extract of urine adsorbed on Al_2O_3. *H*, histamine. Doses in c.c. urine and µg. histamine acid phosphate.

Fig. 5. Action of the same extract of human urine as in Fig. 4 on the rectal caecum of the fowl. The histamine equivalent of the urine as determined on the ileum (Fig. 4) and on the caecum is 0·5 µg./c.c.

show the response of the ileum and of the rectal caecum to the administration of the extracts at different stages of their preparation.

The effect of adsorption on Al_2O_3 differs from one sample to another. When the histamine equivalent is high as in sample 1 adsorption is not necessary for tests made on the guinea-pig's ileum. The assays made on the two test objects show good agreement.

The method of direct hydrolysis and of charcoal adsorption can both be used for quantitative estimation of total histamine in urine. The final extracts prepared by the second method are less contaminated with impurities. On the other hand, a certain loss may occur during the release of the conjugated histamine by acidulated alcohol. The recovery, therefore, depends on the thoroughness with which the charcoal has been extracted.

Urine extracts prepared by the method of direct hydrolysis contain the unknown stimulating material which was described as present in the charcoal filtrate. This does not materially interfere with the assay, since the sensitivity of the ileum to this material rapidly declines. The charcoal method is to be preferred when the histamine equivalent of the urine is low. A larger amount of urine is then used so as to make the final extract more concentrated. The accuracy of the two methods was tested by estimations of urine histamine and of histamine added to water, Tyrode solution or urine. Some of the results are given in Table 4.

TABLE 4. (A), determination of the histamine equivalent of urine and recovery of added histamine by the method of direct hydrolysis and (B), by the method of charcoal adsorption. All the results are given in μg./c.c. of histamine acid phosphate.

Medium	Histamine added	A Recovery by direct hydrolysis	B Recovery by charcoal adsorption	A Percentage recovery by direct hydrolysis	B Percentage recovery by charcoal adsorption
Water	0·20	0·19	0·18	95	90
	0·50	0·50	0·45	100	90
Tyrode	0·50	0·46	0·46	92	92
	2·00	1·80	1·70	90	85
Human urine 1	—	0·25	0·20	—	80
	0·50	0·80	0·70	110	100
Human urine 2	—	1·55	1·40	—	90
	0·50	2·10	1·80	110	80
Dog's urine 3	—	6·20	5·80	—	94
	5·00	11·00	10·40	96	92
Human urine 4	—	0·30	0·25	—	83
Human urine 5	—	1·50	1·40	—	93
Dog's urine 6	—	8·00	8·00	—	100
Dog's urine 7	—	4·30	3·90	—	90

Table 4 shows that the recovery of histamine added to water, Tyrode solution or urine and the determination of the total histamine present in urine extracts is satisfactory with either of the two methods. The method of direct hydrolysis gives somewhat higher results, which are probably due to a small loss of the active substance on the adsorbent.

Conjugated and free histamine. When free histamine is present in the urine it can be quantitatively determined in extracts prepared by the charcoal method before they are hydrolysed. The total histamine is then determined by a second assay after hydrolysis of the extract. The difference between the two results represents the conjugated histamine of the sample. As a check the total histamine can be determined once more by the method of direct hydrolysis. It will be seen later that free histamine is normally present in the urine of some species or may appear in the urine under some experimental conditions.

THE HISTAMINE EQUIVALENT OF THE URINE OF DIFFERENT SPECIES OF ANIMALS

We should like to express our thanks to Dr Kadry, Director of the Cairo Zoological Garden, for the facilities which he placed at our disposal for the collection of urine samples from various animals. When possible, 24 hr. samples were collected, but most urines were taken as single samples either by catheter or during micturition. A few urines were collected from anaesthetized animals directly from the bladder. The samples were collected without regard to the previous history of the animal in respect to the time of feeding, etc. The results are grouped according to whether the animals are (*a*) typical Herbivora, (*b*) animals on varied diet, or (*c*) typical Carnivora. The number of animals used for each species is given in brackets. No number is given for man, dog and rat, since these served for our routine experiments.

In the group of Herbivora the urine of the following species was analysed: rabbit (12), horse (5), donkey (4), water buffalo (5), elephant (3); camel (5), llama (2), anthropoid apes (2). The extreme variations of the urine histamine of these species are between 0·02 and 0·2μg./c.c. histamine acid phosphate; in one horse we found 0·4μg./c.c. There is no appreciable difference between the urines of the different species belonging to this group. The histamine equivalent is uniformly low. Amongst the animals on varied diet the variations in the urine histamine is very considerable, the extreme values for the white rat are 0·1–10·2, the cat (8) 0·3–15·0, the dog 0·2–45·0 and for man 0·1–6·2μg./c.c. In the group of typical Carnivora the variations are again less but the histamine equivalent of all the urines is uniformly high, for the lion (6) 10–15, tiger and leopard (6) 25–40 and the cheetah (1) 15μg./c.c. Urines with a histamine equivalent below 0·2μg./c.c. were analysed by the charcoal method, those of a higher equivalent by the method of direct hydrolysis.

The obvious conclusion from these observations is that the excretion of histamine in the urine depends on the character of the diet. No relation seems to exist between the histamine equivalents of the blood and urine. In the rabbit in which the blood histamine is high there is very little histamine in the urine; in the cat in which the blood histamine is extremely low the histamine of the urine may be as high as 15 μg./c.c.

Carnivora and Herbivora differ not only in respect of the concentration of histamine excreted in the urine but also the form in which it is excreted. In Carnivora almost the entire histamine (98–100%) is excreted in the conjugated form, while in Herbivora most if not the whole of it is free. In view of the very low histamine content and the frequent presence of relaxing substances which are not completely removed by Al_2O_3 we did not analyse the urine of Herbivora any further.

The urine of the rat occupies an intermediate position; free and conjugated histamine are present together but in different proportions. Frequently 40–50% of the histamine is excreted in a free form. In many samples the entire histamine is free. Small amounts of free histamine are also found in most samples of human urine.

EFFECT OF DIET ON THE EXCRETION OF HISTAMINE

The difference in the histamine content of the urine of Herbivora and Carnivora led to the investigation of the effect of administration of meat. The observations were made on rats, dogs and man.

Experiments on rats. Rats weighing 250–300 g. were kept in Hopkins's metabolic cages with the usual precautions and care.

The observations were divided into three periods. During the first the animals were placed on a carbohydrate-fat diet consisting of boiled starch, sugar, olive oil and occasionally some boiled or fried starchy potatoes. During the second period the diet consisted exclusively of slightly roasted or boiled buffalo meat, and during the third period the rats were fed again on a mixture of carbohydrates and fat. The meat was given outside the cages so as not to contaminate the urine. The diets were supplemented by the usual salt mixture, cod-liver oil and clover. The urine was collected in 24 hr. samples, some toluol or thymol being added to prevent putrefaction. Since rats' urine contains conjugated, as well as free histamine, we usually analysed the urine extracts for both, i.e. before and after acid hydrolysis. Urea was analysed by the hypobromite method.

An example of this type of experiment is given in Fig. 6. During the first period of carbohydrate diet the histamine excretion fell to below 10 μg./day, and almost the whole of it was eliminated in a free form. During the next period the total histamine gradually increased to 90 μg., the increase being mainly accounted for by the conjugated histamine. The excretion of free histamine also increased but to a much smaller extent. On return to the carbohydrate diet the excretion of free histamine abruptly diminished to the previous low figures, while the conjugated histamine declined more gradually and almost completely disappeared in the course of 3 days.

Experiments on other rats gave similar results differing only in degree. The maximal elimination of total histamine was in some rats as high as 230 μg., almost the whole of it conjugated. In other animals it did not exceed 50 μg./day. On carbohydrate diet not less and usually more than 50 % of the total histamine was eliminated in a free form.

When rats are kept on a meat diet for a long time their histamine excretion shows fairly wide fluctuations which can be usually traced to the different amounts of meat consumed. As a very rough approximation the elimination of 1 g. of urea corresponds in the rat to the excretion of 100 μg. of total histamine. This relationship is not apparent in 24 hr. samples, but it becomes more obvious with longer periods of observation: for example, a pair of rats on a mixed diet excreted in 18 days 11·6 g. urea and 1070 μg. total histamine. Another pair which received a larger proportion of meat excreted during the same time 23·2 g. urea and 2234 μg. histamine.

The time relations of the excretion of histamine and urea are not the same. The changes in the excretion of histamine lag behind those of urea. In some animals this is so considerable that it can be noticed in 24 hr. samples of urine (Table 5).

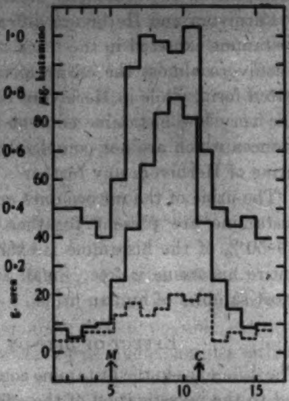

Fig. 6. Effect of administration of meat on the daily excretion of conjugated histamine, free histamine and urea by the rat. From below upwards—free histamine, total histamine in μg. histamine acid phosphate and urea in g./24 hr. The excretion of conjugated histamine is represented by the difference between the total and the free histamine. Meat was given for 6 days between *M* and *C*. Abscissae, days.

Table 5. Showing the effect of a single administration of meat on the daily excretion of urea and of total histamine

Days	Diet	Vol. urine c.c.	Urea g. %	Total histamine μg./c.c.	Excretion of urea g.	Excretion of histamine μg.
1	Potatoes	18	0·6	0·5	0·11	9·0
2	Potatoes	16	0·5	0·7	0·08	11·2
3	Potatoes	20	0·5	0·6	0·10	12·0
4	Potatoes with 29·5 g. meat	20	7·8	2·5	1·56	50·0
5	Potatoes	14	3·0	5·0	0·42	70·0
6	Potatoes	18	0·8	2·5	0·14	45·0
7	Potatoes	10	0·8	1·1	0·08	11·0
				Total	2·49	208·2

The maximal excretion of urea occurred in the above experiment on the day of administration of the meat, and that of histamine on the following day. On the third day the excretion of urea returned to the original level while that of histamine was still increased. Since a more exact determination of these time relations could not be easily made on the rat the experiments were continued on dogs.

Experiments on dogs. The observations were made on female dogs weighing 14–16 kg. As a preliminary an incision was made in the perineal region to facilitate catheterization. Urine samples were collected at intervals of 0·5–4 hr. and analysed for total histamine and urea. Determinations of free histamine were made only occasionally, since it was shown that dogs in common with other Carnivora do not excrete free histamine even when fed on meat.

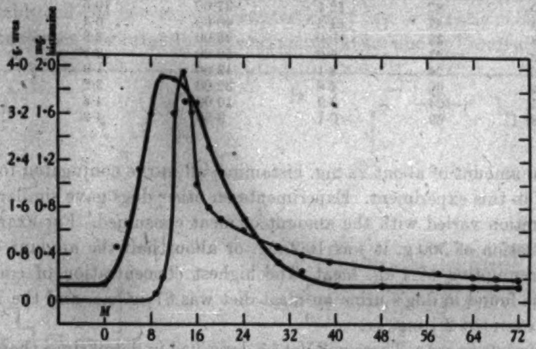

Fig. 7. Effect of administration of 1 kg. of meat on the excretion of conjugated histamine and urea by the dog. Thick line, urea, g.; thin line, conjugated histamine, mg. histamine acid phosphate. Abscissae, hr. The meat was administered at *M*.

In the experiment the results of which are given in Fig. 7, the animal was deprived of food for 36 hr. and then given a large meal of raw minced buffalo meat. The excretion of urea began to increase within 2 hr. after the meal; the maximum was reached on the 10th hour after which the amount of urea gradually dropped to the pre-feeding level in 34 hr. The excretion of histamine began to increase only 6 hr. after the meal and reached a maximum about 4 hr. later than urea. The decline of histamine excretion was at first more rapid and then much slower than that of urea, so that the pre-feeding level was not quite reached even 72 hr. after the meal. Altogether about 20 mg. of histamine were excreted, the entire histamine being present in the conjugated form.

Table 6 is computed from another similar experiment to show the changes in the concentration as well as in the total excretion of histamine and urea following administration of meat.

TABLE 6. Dog, 16 kg. Effect of administration of 1 kg. of minced meat on the excretion of histamine and urea and on their concentration in the urine. During the first 36 hr. after feeding the urine samples were collected at intervals of 4 hr. and then at 8 and 12 hr. Histamine in μg. of histamine acid phosphate.

Hours before and after feeding	Vol. urine c.c.	Urea g. %	Histamine μg./c.c.	Total urea excreted g.	Total histamine excreted μg.
8–4	32	4·8	0·40	1·5	13
4–0	26	6·0	0·35	1·6	9
Administration of 1 kg. meat					
0–4	53	10·7	0·26	5·7	14
4–8	89	12·6	1·40	11·2	125
8–12	127	12·6	26·00	16·0	3302
12–16	115	11·8	53·00	13·6	6095
16–20	87	12·1	37·00	10·5	3219
20–24	57	11·3	40·00	6·4	2280
24–28	33	10·0	43·00	3·3	1419
28–32	27	9·0	50·00	2·4	1350
32–36	24	8·1	43·00	1·9	1032
36–44	66	4·8	32·00	3·2	2112
44–56	124	3·9	10·00	4·8	1240
56–68	69	6·1	3·50	4·2	242

A total amount of about 22 mg. histamine, all in the conjugated form, was excreted in this experiment. Experiments on other dogs gave similar results. The excretion varied with the amount of meat consumed. For example, on administration of 500 g. it was 10·2 mg. or about half the amount excreted after consumption of 1 kg. meat. The highest concentration of conjugated histamine found in dog's urine on meat diet was 87 μg./c.c. and the maximal amount excreted 2·1 mg./hr.

Experiments on man. These will not be described in detail since they present no points of interest beyond those established for the dog. One subject (G.V.A.) excreted on a mixed diet 23–25 g. urea and 1·2–1·4 mg. histamine per day. On the fourth day of a strictly carbohydrate-fat diet the subject excreted 7·5 g. urea and 190 μg. histamine. The excretion of urea declined more rapidly than that of histamine. Beginning from the fifth day the subject was given large amounts of roasted meat. The excretion of urea increased rapidly reaching a maximum of 38 g. on the first day of the meat diet. The excretion of histamine increased more gradually taking about 2 days to reach 2·4 mg. in 24 hr. Almost the whole histamine was excreted as conjugated histamine. Traces of free histamine were found in all the samples of human urine, but they were too small for accurate estimation. The relation between histamine and urea excretion in the dog is higher and in man lower than in the rat. Apparently the facility with which conjugated histamine is formed or excreted differs, being highest in the dog, smaller in the rat and least in man.

Effect of diuresis. Histamine excretion bears no relation to urine flow. This becomes evident after administration of large amounts of water or of urea.

In one experiment a dog on a mixed diet secreted during 4 consecutive hours 15–28 c.c. urine and 190–250 µg. histamine/hr. After administration of 20 g. urea in 100 c.c. milk the urine flow was 81 c.c. during the first and 118 c.c. during the second hour and the elimination of histamine 243 and 206 µg. respectively. Similar results are obtained with animals which are kept on a meat-free diet. For example, in another dog, the urine flow increased from 12 to 95 c.c./hr., while the histamine excretion remained almost the same, 2·2 and 3·8 µg./hr. respectively. Experiments on rats and man gave the same results.

The origin of conjugated histamine

Effect of administration of histamine-free food. The experiments with different diets established the fact that administration of raw or slightly cooked meat greatly increases the excretion of conjugated histamine in Carnivora and in man and also, but to a much smaller extent, the excretion of free histamine in the rat. The experiments do not throw any light on whether conjugated histamine derives from the protein component of meat or from its extractive substances.

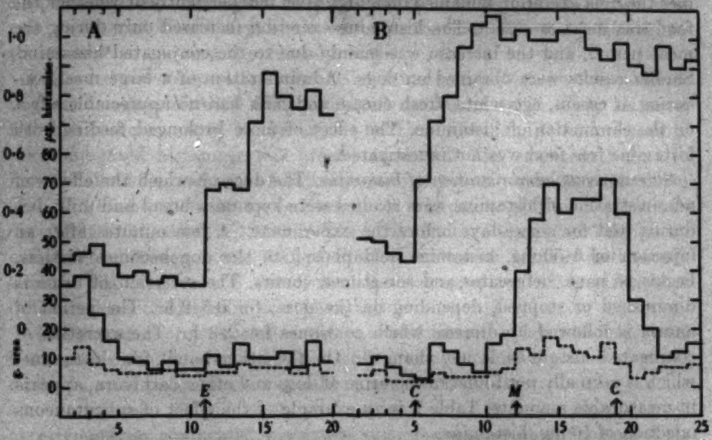

Fig. 8. A. Effect of administration of egg-white on the excretion of histamine and urea by the rat. At *E* the carbohydrate-fat diet was changed to egg-white. B. Effect of administration of casein and of meat to the same rat as in A. At the first *C* the carbohydrate-fat diet was changed to casein, at *M* to meat and at the second *C* again to casein. From below upwards; free histamine, total histamine in µg. histamine acid phosphate and urea in g. Abscissae, days.

This question assumes special importance, since all samples of buffalo meat which we analysed contained histamine, varying from 6 to 20 µg./g. The effect of administration of meat was, therefore, compared with the administration of histamine-free proteins and with the administration of histamine acid phosphate.

The estimation of histamine in the food was made after extraction with trichloracetic acid following the method of Barsoum & Gaddum [1935]. The food extracts were assayed for free and conjugated histamine by testing the extracts before and after hydrolysis in HCl. The histamine equivalent of the hydrolysed and non-hydrolysed extracts of meat was found to be always the same, which suggests that the entire histamine of meat is probably present in a free form. On the other hand, casein, fresh white cheese and egg albumen were found to contain neither free nor conjugated histamine.

Fig. 8 A and B shows the effects of feeding rats on egg albumen and on casein. In Fig. 8 B the rats were fed for 7 days on casein; during the following 7 days they were given meat and then again casein. While on the protein diet the urea excretion remained on a high level independently of whether the food was meat or casein. The histamine excretion increased only during the meat period, and the increase was mainly due to the conjugated histamine. Similar results were obtained on dogs. Administration of a large meal consisting of casein, egg white, fresh cheese and milk had no appreciable effect on the elimination of histamine. The effect of more prolonged feeding with histamine free food was not investigated.

Subcutaneous administration of histamine. The dogs on which the effects of administration of histamine were studied were kept on a bread and milk diet during and for some days before the experiment. A few minutes after an injection of 6–50 mg. histamine acid phosphate the dog becomes restless, begins to pant, defaecates and sometimes vomits. The secretion of urine is diminished or stopped, depending on the dose, for 0·5–2 hr. The period of anuria is followed by diuresis which continues for 2–3 hr. The excretion of conjugated histamine is not changed. On the other hand, free histamine, which is normally not found in the urine of dogs and other Carnivora, appears in measurable amounts. Table 7 is an example of the effect of subcutaneous injection of 10 mg. histamine.

TABLE 7. Dog, 14 kg.; 10 mg. histamine acid phosphate in 5 c.c. saline were injected at the beginning of the third hour.

Time hr.	Urinary secretion c.c./hr.	Conjugated histamine μg./hr.	Free histamine μg./hr.	Total histamine μg./hr.
1	23	26·5	—	26·5
2	18	21·6	—	21·6
3	0	—	—	—
4	16	32·0	107·2	139·2
5	54	30·0	37·8	67·8
6	105	25·1	20·5	45·6
7	99	18·9	9·9	28·8
8	34	20·2	—	20·2
9	25	19·8	—	19·8
10	19	27·8	—	27·8

In this experiment injection of 10 mg. histamine led to an excretion of 175 μg. free histamine. We find no relation between the amount of histamine injected and excreted in the urine. With doses varying from 10 to 50 mg. histamine the amount excreted did not exceed 200 μg. In fact, with the larger doses it was usually less. The excretion of free histamine seems to be related to the duration of the period of anuria which follows the injection, more histamine being excreted in those experiments in which the suppression of urine flow is short. In some experiments with long periods of anuria hardly any free histamine appeared in the urine. For example, injection of 50 mg. histamine suppressed the urine flow in one dog for 60 min. and in another for 150 min. In the first the amount of free histamine excreted was 126 μg., while in the second only traces appeared in the urine. It is probable that most of the injected histamine is destroyed in the animal during the prolonged suppression of the urine. When the period of anuria is short some free histamine escapes through the kidney, but even under the most favourable conditions the amount so excreted is extremely small.

Oral administration of histamine. Histamine acid phosphate was given to dogs in amounts of 0·005–0·5 g. dissolved in 100 c.c. milk. Only traces of free histamine appeared in the urine. On the other hand, the excretion of the conjugated histamine was greatly increased. The excretion begins to increase about 1 hr. after the administration; it gradually reaches a maximum in 7–14 hr. and then slowly declines to its original level, which it reaches, depending on the dose, in 12–60 hr. The total amount of conjugated histamine excreted is between 3 and 5% of the free histamine administered to the animal. Fig. 9 shows the rate of the excretion after oral administration of different doses of histamine acid phosphate.

Administration of conjugated histamine. Subcutaneously injected conjugated histamine is almost quantitatively excreted by the kidneys in an unchanged form. In most experiments we could recover from the urine 85–100% of the amount injected. It will be shown in a later communication that in these experiments no conjugated histamine can be detected in the blood. This indicates that the excretion of conjugated histamine proceeds at the same rate as its absorption and that the kidney threshold to it is very low. Injection of conjugated histamine neither diminishes nor suppresses the urinary secretion probably because, unlike free histamine, it has no vasomotor action. The excess excretion of conjugated histamine begins almost immediately after the injection, reaching a maximum in 2–3 hr. and being completed in about 20 hr. (Fig. 9). The maximal concentration of conjugated histamine observed in the urine was 145 μg./c.c. and the maximal amount excreted 2·50 mg./hr.

Oral administration of conjugated histamine leads to a much greater and more rapid excretion of it in the urine than administration of histamine acid phosphate. Between 50 and 60% of the amount administered is excreted in an unchanged form.

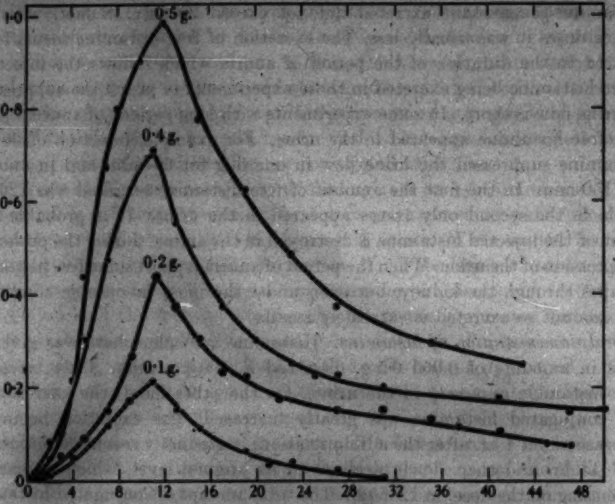

Fig. 9. Excretion of conjugated histamine by the dog after oral administration of histamine acid phosphate. Abscissae, hr.; ordinates, conjugated histamine in mg. histamine acid phosphate. The amounts of histamine administered are given on the figure. The total amounts excreted in the urine were 3·4, 6·4, 16·2 and 24·5 mg. from below upwards.

Comparison between the administration of conjugated and free histamine. In order to make a quantitative comparison between the administration of conjugated and free histamine and of meat, experiments were made in which these substances were administered to the same dog in amounts of approximately equal histamine content. In the series of experiments given in Table 8

TABLE 8. Showing the effect of administration of free and conjugated histamine and of meat on the histamine excretion

Substance and mode of administration	Excess histamine in urine µg.	Duration of excretion hr.	Remarks
Conjugated histamine by subcutaneous injection, 4·5 mg.	4365	9	All conjugated
Histamine acid phosphate by subcutaneous injection, 4·5 mg.	100	2	All free
Conjugated histamine orally, 4·5 mg.	2345	10	All conjugated
Histamine acid phosphate orally, 4·5 mg.	244	14	All conjugated
400 g. of ox meat containing 4·5 mg. histamine	3794	45	All conjugated

all substances were administered in amounts containing 4·5 mg. of histamine acid phosphate or its equivalent.

Table 8 shows the main points of interest concerning the excretion of conjugated and free histamine on their subcutaneous or oral administration. In addition, it shows that the amount of conjugated histamine excreted after administration of meat cannot be accounted for by the 4·5 mg. of histamine of the meat. Had the histamine of meat been the only source of histamine in the urine we would expect an excess excretion of not more than 5% of the amount administered or about 240 μg. instead of 3794 μg. The investigation of this point is being continued.

Discussion

The experiments described in this communication show that histamine is a normal constituent of urine of all the animals which have been so far investigated. In Herbivora it is excreted in a free form and in small amounts. In Carnivora it is excreted in a conjugated and inactive form from which free histamine can be released by hydrolysis.

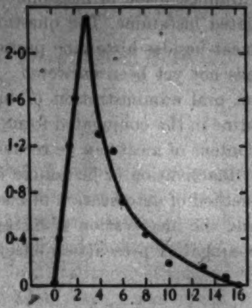

Fig. 10. Excretion of conjugated histamine by the dog after subcutaneous administration of an amount of conjugated histamine equivalent to 10 mg. histamine acid phosphate. Abscissae, hr.; ordinates, conjugated histamine in mg. histamine acid phosphate. The total amount excreted was 9·05 mg.

The amount of conjugated histamine excreted in 24 hr. by the dog varies, depending on the diet, from less than 0·1 mg. to over 30 mg. of histamine acid phosphate, while the concentration of histamine acid phosphate may reach 100 μg./c.c. Subcutaneously injected histamine is excreted in the urine in negligible traces and in a free form; none is converted into the conjugated form. Orally administered histamine is excreted in the urine in a conjugated form to an extent not exceeding 5% of the amount administered. These results indicate that most of the injected histamine is destroyed or fixed in the body, the elimination by the urine playing an unimportant role. On the other hand, a part of the histamine which is absorbed from the alimentary tract is conjugated and excreted in the urine in an inactive form.

The possibility is not excluded that the conjugation of histamine takes place in the kidney although the experiments with injections of histamine point to the intestine or the liver as the more likely organs responsible for the formation of conjugated histamine. No conjugated histamine could be detected in the blood during histamine absorption, which places this substance amongst the low threshold bodies. This is confirmed by the fact that subcutaneously injected conjugated histamine is quantitatively excreted in the urine in a few hours (Fig. 10). On oral administration about 60% of conjugated histamine is excreted in the urine.

Administration of meat greatly increases the excretion of conjugated histamine. The source of it is most likely the histamine present in the meat itself. Administration of histamine-free proteins do not lead to excretion of conjugated histamine. The question whether some other extractive substances of meat besides histamine participate in the formation of conjugated histamine has not yet been answered. Experiments now in progress may explain why on oral administration of histamine not more than 5% is excreted in the urine in the conjugated form, while a much larger proportion of the histamine content of meat can be recovered as conjugated histamine from the urine.

Inactivation of histamine by conjugation must be considered as the third method of detoxication of histamine. Together with the action of histaminase and the inactivation of histamine by the corpuscular elements of the blood, conjugation presents an effective method of dealing with this substance.

SUMMARY

1. A method is described for quantitative estimation of histamine in urine.

2. Histamine can be eliminated in a conjugated and a free form. Carnivora excrete mainly the conjugated and Herbivora the free form. In the rat both forms are present in varying proportions.

3. Administration of meat leads to a considerable increase in the excretion of conjugated histamine.

4. Orally administered histamine is eliminated in a conjugated form to the extent of about 5% of the amount administered. Injected histamine is eliminated in traces in a free form.

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CATION ANTAGONISM IN BLOOD COAGULATION

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The antagonistic effect of ions on vital processes in cells is in many cases connected with the selective permeability of the cell membrane. However, ion antagonism may also conceivably occur in chemical processes taking place inside the cell or on its surface. An example of this may be found in the effect of Ca^{++} and Mg^{++} on the catalysis by myosin of the breakdown of adenosinetriphosphate to adenosinediphosphate and inorganic phosphate. Ca^{++} was found by Needham [1942] and Bailey [1942] to be an activator of this system. Mg^{++} was stated by these authors to have no effect, although Ljubimova & Pevzner [1941] had reported some inhibition. This apparent contradiction was explained by the finding that Mg^{++} suppresses the Ca^{++} activation [Greville & Lehmann, 1943]. The inhibition of adenosinetriphosphatase by Mg^{++} was observed by Ljubimova & Pevzner [1941] because they used calcium adenosinetriphosphate as substrate, whereas in the experiments of Needham [1942] and Bailey [1942] Ca^{++} was absent since the sodium salt was used.

Mg^{++}/Ca^{++} antagonism in an enzyme system is not unique for myosin, but has also been demonstrated in adenosinetriphosphate breakdown by extracts of the electric organ of *Torpedo* [Greville & Lehmann, 1943]. Indeed, in this instance the role of the ions is reversed, Mg^{++} being the activator [Bailey, 1939] and Ca^{++} its antagonist.

In the present communication, the coagulation of blood is brought forward as another process in which a Mg^{++}/Ca^{++} antagonism may be demonstrated. Blood clotting is activated by Ca^{++} [Green, 1887; Ringer & Sainsbury, 1890]; and it is well known that Mg salts, like other neutral salts, prevent coagulation. The latter effect has not hitherto been considered in relation to the part played by Ca^{++}, but has been regarded as due to a physico-chemical stabilization of fibrinogen [Quick, 1942], or alternatively to a preservation of the platelets from disintegration [Best & Taylor, 1943]. We have found, however, that the inhibition of clotting by $MgCl_2$, in some concentrations at least, is counteracted by the addition of $CaCl_2$. In addition, we have demonstrated an antagonism between Ca^{++} on the one hand and Ba^{++}, Mn^{++}, Na^{+} and K^{+} on the other.

METHODS

Removal of blood. Blood was taken from the antecubital veins of about twenty-five human subjects. Care was taken not to use the same vein twice. The subjects, who were all in good physical condition, were of both sexes and of ages ranging from 20 to 70 years. 20 ml. syringes were used, with 17-gauge serum needles.

Experiments with whole blood. A dry syringe was used and the blood immediately measured out into the tubes in which the clotting-time determinations were made, and which already contained the various salt solutions.

Experiments with plasma. Like blood, plasma was obtained without the use of an anticoagulant. We adhered rigorously to the following technique: a syringe coated with liquid paraffin was used, and the blood immediately transferred to paraffined centrifuge tubes immersed in ice. The tubes, still surrounded by ice, were left in the refrigerator for 10 min. They were then centrifuged for 10 min. at 2500 r.p.m. The plasma was removed with a paraffined teat pipette into another paraffined vessel. It was then immediately measured out with a 1 ml. bulb pipette into the tubes containing the salt solutions. As will be seen in the text, in some experiments plasma was diluted with an equal volume of distilled water before being pipetted.

Determination of clotting time. The tubes in which the clotting times were determined were 75 mm. long with a diameter of 13 mm. They were of soda glass, except in the experiment of Table 1 in which Pyrex tubes were used. The tubes were tilted at appropriate intervals to detect clotting. The time at which the surface became immobile was taken as 'clotting time'. Sometimes, when clotting was greatly protracted, the shrinkage of the fibrin meshwork prevented the clot from ever completely filling the fluid. When this occurred, the time at which the clot occupied the maximum volume (80–90% of the total) was taken as clotting time.

The experiments were carried out at room temperature. The initial temperature lay always between 20 and 23° C.

AnalaR reagents and glass-distilled water were used throughout.

RESULTS

Mg^{++} *and whole blood.* When neutral salts such as Na_2SO_4 or NaCl are used for the prevention of clotting, it is customary to add a strong solution to give a final concentration of about 1 *M*. With $MgSO_4$ the final concentration is usually 0·2 *M* [Best & Taylor, 1943]. However, it will be seen from Table 1 that $MgCl_2$ at one-fifth of the latter concentration prevents clotting, and considerably delays it at one-tenth.

These findings may possibly be of practical value for the storage of blood. It was found that if the effective concentration of about 0·05 *M* was obtained

TABLE 1. The anticoagulant effect of $MgCl_2$ on whole blood

The tubes contained 2 ml. whole blood with various vol. of *M* $MgCl_2$ solution as shown below. 25° C.

Tube no. ...	1	2	3	4	5	6
Vol. of *M* $MgCl_2$ added (ml.)	0	0·03	0·05	0·08	0·10	0·20
Final concentration of added Mg^{++} (*M*)	0	0·015	0·024	0·038	0·048	0·09
Clotting time ($\infty = >24$ hr.)	5 min.	25 min.	7 hr.	∞	∞	∞

by adding 1 vol. of *M*/7 $MgCl_2$ to 2 vol. of blood, no clotting and no appreciable haemolysis were observed after 1 week's storage in the refrigerator. The cells remained morphologically undamaged. If, in order to reduce the dilution of the blood, $\frac{1}{2}$ vol. of a $MgCl_2$ solution of twice the above strength was added to 2 vol. of blood, very slight haemolysis occurred.

Mg^{++} *and plasma.* Since in whole blood the complicating factor of distribution of ions between cell water and plasma water arises, it is better to study ion antagonism in plasma. Table 2 shows the influence of various concentrations of $MgCl_2$ on the clotting of plasma. The minimum concentration effectively preventing clotting was of the same order as in Table 1.

TABLE 2. The anticoagulant effect of $MgCl_2$ on plasma

Room temperature, initially 23° C. In each tube, 1 ml. plasma + 0·1 ml. $MgCl_2$ solution (except in control with water). The concentrations of the added $MgCl_2$ solutions are shown in column 2.

				Appearance		
	$MgCl_2$ (*M*)	Final molarity of added Mg^{++}	Clotting time	After 1 day	After 7 days	After 12 days
1	(H_2O)	—	13 min.	—	—	—
2	0·1	0·009	15 min.	—	—	—
3	0·2	0·018	27 min.	—	—	—
4	0·3	0·027	75 min.	—	—	—
5	0·4	0·036	∞	Clear	*A few threads*	*A few threads*
6	0·5	0·045	∞	Clear	Clear	*A few threads*
7	0·6	0·055	∞	Clear	Clear	Clear

The effect of Mg^{++} in higher concentrations than those in the above table, up to a final molarity of 0·73, was also tested. The result was throughout the same as in no. 7.

Reversibility. It is well known that 'salted blood' or 'salt plasma' clots on mere dilution. Dilution of course reduces the concentration of Ca^{++} as well as of Mg^{++}. We have found that the clotting of plasma, which has been kept fluid by $MgCl_2$, is induced on the addition of water with greater certainty if $CaCl_2$ is added as well (Table 3).

Antagonism. Although in the above experiment Ca^{++} clearly accelerated clotting in the presence of Mg^{++}, this does not allow us to postulate an antagonism between these ions. Owing to the dilution of the plasma an increase in Ca^{++} concentration would have accelerated clotting even if no Mg^{++} had been added. To demonstrate a true antagonism more is needed. A counteraction of the Mg^{++} inhibition by the addition of Ca^{++} would have to be obtained in plasma in which the Ca^{++} concentration is already optimal, i.e. is sufficient to produce the maximum rate of clotting in the absence of inhibitors.

TABLE 3. Clotting of $MgCl_2$-treated plasma following dilution and the addition of $CaCl_2$

1·75 ml. plasma was diluted with 1·75 ml. water and 0·18 ml. *M* $MgCl_2$, and allowed to stand 1 day at room temperature. At the end of this period there were no signs of clotting, and 1 ml. of the mixture was placed in each of three tubes, with additions as below.

Tube no. ...	1	2	3
Water (ml.)	1·0	—	1·0
$CaCl_2$, 0·033 *M* (ml.)	—	0·1	0·1
Appearance 1 hr. later	Slight clotting	Fluid	*Clotted*
Appearance after further 21 hr.	Slight clotting	Fluid	—

At this time further additions were made, as below.

Tube no. ...	1	2
Water (ml.)	—	1·0
$CaCl_2$, 0·033 *M* (ml.)	0·1	—
Appearance 2 hr. later	*Clotted*	Very slight clotting
Appearance after further 8 hr.	—	*Clotted*

This condition was fulfilled in the experiments in Table 4. In plasma diluted with $\frac{1}{5}$ vol. of water the Ca^{++} concentration was still optimal. Thus, further addition of Ca^{++} did not accelerate clotting in absence of inhibitor, but at the same time it counteracted the inhibitory effect of Mg^{++}.

The normal Ca concentration of plasma is about $2{\cdot}5 \times 10^{-3}$ *M*. It will be seen that increasing the Ca content up to double by the addition of $CaCl_2$ did not alter the clotting time (Table 4 A, B). Raising the concentration four times even resulted in a slight lengthening of the clotting time (Table 4 C). This retardation of clotting by high concentrations of Ca^{++} is of course well known [Horne, 1896].

TABLE 4. Mg^{++}-Ca^{++} antagonism in blood clotting

Room temperature. In each experiment four tubes were used, each containing 1 ml. plasma. Further additions were as follows:

Control:	0·2 ml. water.
Ca:	0·1 ml. $CaCl_2$ solution + 0·1 ml. water.
Mg:	0·1 ml. $MgCl_2$ solution + 0·1 ml. water.
Mg + Ca:	0·1 ml. $MgCl_2$ solution + 0·1 ml. $CaCl_2$ solution.

The concentrations of the $CaCl_2$ and $MgCl_2$ solutions added varied from experiment to experiment. The final concentrations of Ca and Mg are shown, allowance being made in the case of Ca for the amount already present in the plasma. A similar allowance is not necessary for Mg because relatively high concentrations were used.

The experiments fall into three groups:

A, lower Mg concentration with low Ca concentration.

B, higher Mg concentration with medium Ca concentration.

C, higher Mg concentration with high Ca concentration.

∞, no clotting in 24 hr. ∞′, some fibrin formation in 24 hr.

Group	Exp. no.	Mg concentration ($M \times 10^{-2}$)	Ca concentration ($M \times 10^{-3}$)	Clotting time (min.)			
				Control	Ca	Mg	Mg + Ca
A	1	2·8	3·7	16	16	∞	125
	2	2·8	3·7	8	8	70	43
	3	2·8	3·7	11	11	80	40
B	4	3·3	4·8	15	15	∞′	136
	5	3·3	5·0	13	13	∞	135
	6	3·3	4·7	12	12	∞	335
C	7	3·3	10·4	15	20	∞′	161
	8	3·3	10·6	13	15	∞	510
	9	3·3	10·3	12	13	∞	∞′

Table 4 thus provides the necessary evidence that there is an antagonism between Mg^{++} and Ca^{++} with respect to plasma clotting. It is now to be expected that the amount of Ca^{++} which has to be added to a given volume of plasma in order to produce a maximum rate of clotting will increase with the amount of Mg^{++} present. This was found to be the case (Table 5). Diluted plasma was prevented from clotting by three different concentrations of added Mg^{++}: $3{\cdot}3 \times 10^{-3}$ *M*, $4{\cdot}2 \times 10^{-3}$ *M*, and $5{\cdot}0 \times 10^{-3}$ *M*. $CaCl_2$ was added in various amounts, the final Ca^{++} concentration ranging between *ca.* 1×10^{-3} and 10×10^{-3} *M*. It will be seen that increasing the Ca^{++} concentration by about one-half induced clotting in the samples containing the two lower concentrations of Mg^{++}, but at the highest Mg^{++} concentration raising the Ca^{++} concentration to over double the original value was ineffective. Clotting was, however, obtained at about 3½ times the initial Ca^{++} concentration. Furthermore, at the lowest Mg^{++} concentration a maximal clotting rate appears to have been reached with about 2×10^{-3} *M* Ca^{++}, at the medium Mg^{++} concentration with about $2{\cdot}5 \times 10^{-3}$ *M* Ca^{++}, and at the highest Mg^{++} concentration with about 4×10^{-3} *M* Ca^{++}.

TABLE 5. The counteraction of Mg^{++} inhibition by Ca^{++}: the need of a higher Ca concentration with rising Mg^{++} concentration

In each tube, 1 ml. of 1:1 dilution of plasma in water + 0·1 ml. $MgCl_2$ solution + 0·1 ml. $CaCl_2$ solution or water. The strength of the $MgCl_2$ and $CaCl_2$ solution varied from tube to tube. Final concentrations are shown, allowance having been made for the Ca already present in the plasma. Room temperature. ∞ means no clotting in 24 hr.

		Calcium concentration ($M \times 10^{-3}$)						
		1·2	1·8	2·1	2·6	4·0	5·4	7·2
Experiment *A*	Mg concentration ($M \times 10^{-3}$)	Clotting time (min.)						
	3·3	∞	50	*38*	37	36	—	—
	4·2	∞	150, ∞	140	*55*	53	68	68
	5·0	∞	—	∞	∞	*90*, ∞	90, ∞	—
		Calcium concentration ($M \times 10^{-3}$)						
		1·1	1·7	2·0	2·5	3·9	5·3	9·4
Experiment *B*	Mg concentration ($M \times 10^{-3}$)	Clotting time (min.)						
	3·3	∞	50	*41*	39	36	—	—
	4·2	∞	165, ∞	144	*59*	47	—	—
	5·0	∞	—	—	∞	*159*	155	180,700

The above clotting times represent the means of duplicates, except that where the duplicates disagreed both values are given.

Table 6 shows the effect of addition of 3×10^{-3} *M* $CaCl_2$ on the inhibition of the clotting of diluted plasma by various concentrations of $MgCl_2$. In most

Table 6. The range of $MgCl_2$ concentration within which counteraction by $CaCl_2$ can be demonstrated

In each tube, 1 ml. of 1:1 dilution of plasma in water + 0·1 ml. $MgCl_2$ solution + 0·1 ml. $CaCl_2$ solution or water. Final concentration of added $CaCl_2$ 3×10^{-3} *M* (total Ca concentration about 4×10^{-3} *M*). Room temperature. ∞ indicates that clotting did not occur within 24 hr.

Plasma sample no.	Final concentration of added $MgCl_2$ ($M \times 10^{-2}$)	Clotting time		Reduction of $MgCl_2$ inhibition by added $CaCl_2$
		Without added $CaCl_2$	With added $CaCl_2$	
1	8·3	∞	∞	0
2	6·7	∞	∞	0
3	5·9	∞	∞	0
4	5·0	∞	∞	0
5	5·0	∞	∞	0
6	4·2	∞	40 min.	+
7	4·2	∞	85 min.	+
8	4·2	∞	44 min.	+
1	4·2	∞	114 min.	+
3	4·2	∞	45 min.	+
5	4·2	160 min.	47 min.	+
4	3·3	∞	36 min.	+
5	3·3	∞	36 min.	+
2	3·3	∞	7 hr.	+
3	2·8	45 min.	40 min.	(+)
6	2·8	45 min.	26 min.	+
7	2·8	43 min.	29 min.	+
8	2·1	14 min.	11 min.	(+)

of the experiments $CaCl_2$ was also added in concentrations higher and lower than the above, but in no case was a better counteraction obtained. It will be seen that $CaCl_2$ will counteract the effect of $MgCl_2$ if the concentration of the latter lies below about 5×10^{-2} *M*; with more $MgCl_2$ the inhibition is not affected. It might have been supposed that the effect of these higher concentrations of Mg^{++} would be counteracted if the Ca^{++} concentration were correspondingly increased. That this was not so may be due to the circumstance, already mentioned, that Ca^{++}, in concentrations above an optimum, ceases to activate clotting and becomes inhibitory.

It may be remarked that the concentration of added Ca^{++} which has been found to counteract the inhibition of clotting by Mg^{++}, i.e. 3×10^{-3} *M*, is of the same order of magnitude as the Ca^{++} concentration of plasma.

The effect of cations other than Mg^{++}. Horne [1896], who first showed an inhibitory effect of high Ca^{++} concentrations on blood clotting, found a similar inhibition by Ba^{++} and Sr^{++}. The question arises whether, like Mg^{++}, other cations delay clotting by counteracting the effect of Ca^{++}. Before testing this point, we determined the approximate concentrations at which various salts delayed plasma clotting for about 24 hr. Under our experimental conditions (1 ml. plasma + 0·1 or 0·2 ml. salt solution at room temperature) the necessary final molarities of *added* salt were approximately as follows:

$BaCl_2$	$MnCl_2$	$MgCl_2$	$CaCl_2$	NaCl	KCl
0·015	0·015	0·035	0·065	0·25	0·25

We then tested the effect of $CaCl_2$, added in a concentration which in absence of inhibitors did not accelerate clotting, on the inhibition by $BaCl_2$, $MnCl_2$, NaCl and KCl. Table 7 contains representative experiments, which were amply confirmed. It will be seen that the inhibitory cations can be counteracted by Ca^{++}. As with Mg^{++}, the concentration of inhibitor must be carefully chosen if the antagonism is to be demonstrated. The most suitable concentrations are approximately those which are just sufficient to delay clotting for 24 hr.

TABLE 7. Antagonism between Ca^{++} and Ba^{++}, Mn^{++}, Na^+, K^+

Room temperature. 1 ml. plasma + 0·1 ml. inhibitory salt solution + 0·1 ml. water or 0·033 *M* $CaCl_2$ solution (final concentration of added Ca $2\cdot7\times10^{-3}$ *M*). In the controls, 1 ml. plasma + 0·2 ml. water, or 1 ml. plasma + 0·1 ml. water + 0·1 ml. 0·033 *M* $CaCl_2$ solution. 'Final concentration' refers to added salt, no account being taken of the salts already present in the plasma. ∞, not clotted in 24 hr.

	Inhibitory chloride added			Clotting time	
Plasma sample	Cation	Concentration of solution (*M*)	Final concentration (*M*)	Without added $CaCl_2$	With added $CaCl_2$
A	Control	—	—	15 min.	17 min.
B	Control	—	—	15 min.	15½ min.
C	Control	—	—	13 min.	14 min.
C	Ba^{++}	0·10	0·0084	35 min.	26 min.
B	Ba^{++}	0·15	0·0125	∞	85 min.
B	Ba^{++}	0·20	0·017	∞	∞
B	Mn^{++}	0·10	0·0084	37 min.	30 min.
B	Mn^{++}	0·15	0·0125	16 hr.	73 min.
B	Na^+	2·0	0·17	39 min.	33 min.
B	Na^+	2·5	0·21	90 min.	42 min.
A	Na^+	3·0	0·25	∞	2½ hr.
A	K^+	2·5	0·21	3½ hr.	1½ hr.
A	K^+	2·7	0·225	∞	2 hr.
A	K^+	3·0	0·25	∞	22 hr.

Ringer & Sainsbury [1890] claimed to have shown an antagonism in blood clotting between Na^+ and K^+ on the one hand and Ca^{++} on the other. Their evidence was that when blood was diluted twenty times with NaCl or KCl solutions clotting was greatly delayed, but occurred more rapidly if the salt solutions contained $CaCl_2$. However, when blood is diluted twenty times the Ca^{++} concentration is far below the optimal, and acceleration of clotting would in any case be expected on addition of Ca^{++}. Thus the experiments of Ringer & Sainsbury, although suggestive, give no proof for an antagonism between Ca^{++} and the monovalent cations. The necessary evidence is provided in Table 7.

DISCUSSION

An antagonism between Mg^{++} and Ca^{++} having been established, it becomes necessary to seek a possible mechanism. This question is bound up with that of the normal role of calcium.

Mellanby & Pratt [1940] suggest that Ca^{++} may act *physico-chemically* by antagonizing other cations with respect to the soluble fibrinogen-prothrombin complex, and thus facilitating the thrombin formation. If this suggestion should prove to be correct, the cation antagonisms described in the present paper will receive their explanation on the basis of the principles of colloid chemistry.

It is also widely believed, however, that Ca^{++} activates blood clotting *chemically* by participating in the conversion of prothrombin to thrombin (the Morawitz-Fuld hypothesis). Quick [1942] writes the relevant equations as follows:

$$\text{Prothrombin} + CaCl_2 \rightarrow \text{prothrombin Ca ('active prothrombin')}, \quad \text{(i)}$$

$$\text{Prothrombin Ca} + \text{thrombokinase} \rightarrow \text{thrombin}. \quad \text{(ii)}$$

If the role of Ca^{++} is accurately described by this hypothesis, an explanation of our findings will have to be found in terms of chemical kinetics.

It may be supposed that before reaction (ii) takes place, a complex $XCaY$ is temporarily formed between prothrombin (X), Ca and thrombokinase (Y). Then, as the result of the formation of this particular complex, prothrombin is transformed to thrombin. Mg^{++} will be an inhibitor if it can compete with Ca^{++} for its position in the complex, and if in addition $XMgY$ is more stable than $XCaY$, so that the transformation of prothrombin proceeds more slowly or virtually not at all. The effect of Mg^{++} may then be regarded as a competitive inhibition.

Competitive inhibition is commonly observed in enzyme studies, the inhibitor usually having a constitution similar to that of the substrate. Inhibitor molecules replace the true substrate molecules at the active centres of the enzyme without being able to enter into the catalysed reaction. Hence, to consider the Ca^{++}/Mg^{++} antagonism in the present system as due to competitive inhibition involves no new conception, but merely the extension of a well-known principle to a system in which an ion is an activator by virtue of its being an essential constituent of the reaction complex. Nor can there be objection to extending the principle to a system which is probably non-enzymic [see Quick, 1942]; for once the complex $XCaY$ is formed, it is unimportant whether both X and Y undergo chemical change, or whether one acts as an enzyme.

A tentative explanation may now be offered for the inhibition of clotting by Ca^{++} in supra-optimal concentrations. The complex $XCaY$ would presumably be formed by a successful collision between XCa and Y or between YCa and X. If as the Ca^{++} concentration rises more YCa and XCa are formed, so that less free Y and X are available, the chance of formation of the $XCaY$ complex will be correspondingly reduced.

Similarly, the formation of XMg and YMg, resulting in a reduction of the amount of available free X and Y, would represent a second way in which Mg^{++} could prevent by competition the formation of $XCaY$, and so suppress the Ca^{++} activation.

The inhibition of clotting by the other divalent cations may be explained similarly in terms of competitive inhibition. It is less obvious how the inhibition by the monovalent cations Na^{+} and K^{+} can be due to a suppression of the Ca^{++} activation. However, these ions become inhibitory in considerably higher concentrations than the divalent cations; and they are likely to be enriched in the immediate neighbourhood of the acidic groups with which the

Ca^{++} most probably combines. It may therefore be suggested that they hinder the collisions between Ca^{++} and the reactants which, as we have assumed, necessarily precede reaction.

The adenosinetriphosphatase of myosin, mentioned in the introduction to this paper, has much in common with blood clotting with respect to the effects of cations (Table 8). The formation prior to reaction of a complex between myosin, Ca^{++} and adenosinetriphosphate can be postulated. This may be done with the more confidence, in that the formation in such systems of a complex between enzyme, divalent metal and substrate is now generally assumed [cf. Warburg & Christian, 1941]. The system may then be treated in the same way as blood clotting to explain the Mg^{++}/Ca^{++} antagonism and the inhibition by supra-optimal concentrations of Ca^{++}. The hypothesis that cation antagonism can be regarded as a competitive inhibition may in fact be capable of wider application than to the Ca-activated process of blood clotting.

TABLE 8

	Myosin adenosinetriphosphatase	Blood clotting
1. Ca^{++} activates	Needham [1942] Bailey [1942]	Green [1887] Ringer & Sainsbury [1890]
2. Ca^{++} in supra-optimal concentrations inhibits	Greville & Lehmann [1943]	Horne [1896] v. Zárday [1934]
3. Other divalent cations may activate, but are less effective than Ca^{++}	Bailey [1942] (Ba^{++}, Mn^{++})	Ringer & Sainsbury [1890] (Sr^{++}, Ba^{++})
4. The inhibition by Mg^{++} may be counteracted by the addition of Ca^{++}	Greville & Lehmann [1943]	Present paper

SUMMARY

1. The inhibition by the chlorides of Mg, Ba, Mn, Na and K of the coagulation of human whole blood and plasma, obtained without an anticoagulant, has been studied.

2. Clotting is indefinitely delayed by the addition of $MgCl_2$ to give a final concentration of about 0·04 *M*. This is several times smaller than the concentration of $MgSO_4$ usually specified for the preparation of 'salted blood' and 'salt plasma'.

3. Whole blood may be kept fluid without haemolysis by the addition of $\frac{1}{2}$ vol. of $M/7$ $MgCl_2$.

4. The inhibition of the coagulation of plasma by $MgCl_2$ is, within limits, counteracted by the addition of $CaCl_2$. There is a true antagonism between Mg^{++} and Ca^{++} with respect to plasma clotting.

5. The amount of Ca^{++} which has to be added to a given volume of plasma to produce a maximal rate of clotting increases with the amount of Mg^{++} present.

6. Ba^{++}, Mn^{++}, Na^{+} and K^{+} also show an antagonism towards Ca^{++} with respect to plasma clotting.

7. There is a close similarity between the effects of cations on blood clotting and on the adenosinetriphosphatase of myosin. The mechanism of these effects is discussed.

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THE ISOLATION AND IDENTIFICATION OF A PRESSOR BASE FROM NORMAL URINE

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The pressor base described in this paper is one of the two bases previously shown to be present in the steam distillate from concentrated urine made strongly alkaline with caustic soda [Lockett, 1944]. The pressor base here described is the base A of the previous paper, in which a base of similar properties was shown to be obtained by a lead precipitation method, the base passing into the filtrate. The base A and the base of the lead filtrate were both volatile in steam, were extracted by ether from alkaline solution, and gave oxalates insoluble in ether. In this paper, both these bases are identified as the one base A, and observations leading to the identification of the main C-structure with that of *l*-nicotine are reported; evidence is given that a more active form of this base may exist, closely related to *l*-nicotine.

EXPERIMENTAL

1. *The isolation of the base A as the pure oxalate from the steam distillate of urine concentrate made strongly alkaline with caustic soda*

In the course of 9 weeks, 742 l. of normal human urine were worked up for the isolation of the pure oxalate of the base A. The urine was collected from male patients in the Examination Hall extension of Addenbrooke's Hospital; most of these patients were convalescent and surgical, and the urine from renal cases was excluded. Phenol was used as a bacteriostatic, but no attempt was made to use only sterile urine.

The base was first isolated as the crude oxalate, the crude oxalate was then purified and analysed for the establishment of the empirical formula. The experimental procedure is described under these headings, and is followed by a description of certain changes occurring in a solution of the pure oxalate of the base A in 0·9% NaCl, on keeping.

(a) *The isolation of the crude oxalate*

The steps in the isolation are represented in Scheme I.

SCHEME I

Treatment of urine concentrate each day

Urine concentrate, pH 13·0 (NaOH)
↓
Steam distillate
↓
Blood charcoal
↓
Acid elution

The acid eluates were worked in six groups corresponding to weeks of urine collection

Combined acid eluates for one group, made alkaline (NaOH)
↓
Chloroform
↓
Aqueous acid with reduction in volume, made alkaline
↓
Benzoylated (Schotten-Baumann) and centrifuged [base B precipitated]
↓
Supernatant, made alkaline
↓
Chloroform
↓
Aqueous acid with reduction in volume, made alkaline
↓
Ether, dried
↓
Crude oxalate precipitated, and once recrystallized

Throughout 6 weeks, the urine of the previous 12–24 hr. was concentrated each day to approximately ½ vol. on a large water-bath carrying 21 l., the temperature of the water being 80–85° C., and a good draught provided. The concentrate was cooled overnight, filtered, and then steam distilled in 200 c.c. fractions which had been brought with 20% NaOH to a pH just blue to alizarin used as an external indicator. The distillate, 150–160 c.c. from each 200 c.c. fraction, was poured on to approximately 1 g. blood charcoal (British Drug Houses Ltd.) for each 400 c.c., and was stirred. As the charcoal separated only very slowly, shaking was not necessary. After 24 hr. the charcoal was filtered off, and the filtrate was again treated with charcoal. The charcoal was eluted by boiling and stirring with 2% HCl, the eluates from the second charcoal treatments being added to those of the first. These charcoal eluates were pooled in six groups corresponding to weeks of urine collection, and were stored in the ice chest until the following week, in which they were worked up. The eluates of group 3, however, remained in the ice chest for an additional week, these being then added to those of group 4, and the two worked up together.

The charcoal eluates of a group were made alkaline to litmus with NaOH, and after standing until the precipitate flocculated, were filtered, and then filtered through kieselguhr. The clear colourless solution obtained was extracted four times with chloroform. The combined chloroform extracts were then shaken three times with small volumes of 1% HCl, and the combined acid-aqueous extracts were boiled to remove all traces of chloroform. By this means the total volume for each group was reduced to 120–180 c.c. This solution was neutralized to litmus with 20% NaOH. Solid NaOH was now added gradually, with ice cooling, to 10%, and benzoylation by the Schotten-Baumann method was continued until 0·5 c.c. of the solution no longer gave the positive colorimetric test associated with the base B [Lockett, 1944]. The separation of the precipitate occurred more readily if the solution were not kept too strongly alkaline. The solution was then either filtered or centrifuged. The filtrate or supernatant fluid was warmed in a water-bath at 60° C. for 15 min., cooled, and then thoroughly extracted with chloroform. The combined chloroform extracts were shaken with 10 c.c. 1% HCl three times, and the combined acid extracts were boiled to free them of chloroform, and cooled. After the cooled solution had been made alkaline to litmus with NaOH, it was extracted five times with ether, and the combined ether extracts were dried over anhydrous Na_2SO_4 for several days. The ether solution was then decanted, and treated with a saturated solution of oxalic acid in ether, which was added drop by drop until there was no further increase in the white turbidity which developed. The precipitate was spun down, washed with ether on the centrifuge, and then recrystallized from 98% alcohol, these crystals being the crude oxalate. The oxalate began to separate at once on cooling. A weighed amount of the dried oxalate was dissolved in 0·9% NaCl, and tested for pressor activity by intravenous injection into a cat anaesthetized with chloralose, and prepared in the manner described in the previous paper [Lockett, 1944]. The remainder of the oxalate obtained from each group of charcoal eluates was dissolved in 10 c.c. water, one drop 10% HCl was added, and the solution was stored, after sterilization by boiling, in the ice chest to await the final crystallizations.

The weights of crude oxalate obtained from each group of charcoal eluates, the litres of urine represented, and the pressor responses obtained, are given in Table 1.

TABLE 1

Group	Wt. of crude oxalate mg.	Vol. of urine represented l.	Wt. of cat kg.	Wt. of oxalate injected (crude) μg.	Arterial pressure, mm. Hg	
					Base-line	Rise
I	68·5	116	2·3	70·0	144	32
				140·0	143	56
II	86·25	160	2·4	107·5	158	34
				215·0	159	66
III IV	63·4	234	2·25	25·4	160	40
				51·0	160	54
V	65·0	132	—	Tested in solution before the oxalate was prepared	—	—
VI	48·5	100	—		—	—

The final solution to be used for ether extraction, and precipitation of the crude oxalate of group V eluate, was tested for pressor activity: the crude oxalate was subsequently prepared and weighed but not tested. The volume of this solution was 72 c.c. 0·1 c.c. of this solution when injected intravenously into a cat, wt. 2·25 kg., and under chloralose anaesthesia produced a rise of arterial pressure of 74 mm. Hg from a base-line of 155 mm. Hg. The corresponding solution from group VI, volume 66 c.c., was similarly tested on the same cat, and in a dose of 0·3 c.c., from a base-line of 155 mm. Hg, produced a rise in arterial pressure of 46 mm. Hg.

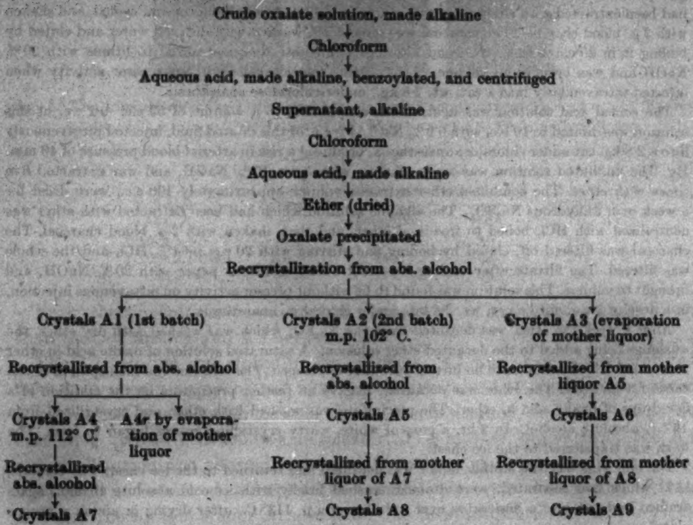
There should, then, have been approximately 6000 pressor doses of the oxalate of the base A in the five 10 c.c. solutions of the crude oxalate.

(*b*) *The purification of the crude oxalate*

The steps are represented in Scheme II. In the purification of the crude oxalates, the base A was passed from an alkaline solution of the whole weight of crude oxalate to chloroform, and thence to aqueous HCl. Benzoylation by the Schotten-Baumann method was repeated to ensure the removal of the whole of the base B which has been shown to benzoylate readily by this method [Lockett, 1944]. The base A was then again passed into chloroform from alkaline solution, and back into aqueous HCl; and was then passed into ether from alkaline solution, and dried in ether. The oxalate was precipitated by the addition of oxalic acid in ether, and was repeatedly recrystallized until crystals of constant melting-point and pressor activity were obtained. The actual procedures will now be described.

Scheme II

The purification of all the crude oxalate from the six groups


Crude oxalate solution, made alkaline
Chloroform
Aqueous acid, made alkaline, benzoylated, and centrifuged
Supernatant, alkaline
Chloroform
Aqueous acid, made alkaline
Ether (dried)
Oxalate precipitated
Recrystallization from abs. alcohol
Crystals A1 (1st batch)
Crystals A2 (2nd batch) m.p. 102° C.
Crystals A3 (evaporation of mother liquor)
Recrystallized from abs. alcohol
Recrystallized from abs. alcohol
Recrystallized from mother liquor A5
Crystals A4 m.p. 112° C.
A4r by evaporation of mother liquor
Crystals A5
Crystals A6
Recrystallized abs. alcohol
Recrystallized from mother liquor of A7
Recrystallized from mother liquor of A8
Crystals A7
Crystals A8
Crystals A9

The 50 c.c. of solution of crude oxalate was made alkaline with 2·5 c.c. 20% NaOH, and was extracted five times with chloroform. The aqueous phase was neutralized to litmus paper with HCl, boiled to free it from chloroform, and was shown to be without pressor activity on intravenous injection into a chloralosed cat, wt. 2·8 kg., in a dose of 5 c.c.

The combined chloroform extracts, having a volume of 120 c.c., were extracted four times with 12–14 c.c. 0·64% HCl. These combined acid extracts were boiled to remove chloroform, cooled, neutralized and then made alkaline with 10 c.c. 20% NaOH, and the solution cooled in ice. A fifth extraction of the chloroform was carried out with 10 c.c. 0·64% HCl, the aqueous phase was boiled, cooled and neutralized with NaOH, and was found to be without pressor activity when injected intravenously into a chloralosed cat, wt. 2·8 kg., in a dose of 1 c.c.

The ice-cold alkaline solution was now benzoylated by the Schotten-Baumann method; a small amount of precipitate separated and was spun down. The process was repeated on the supernatant, but no further precipitate formed. The solution was warmed to destroy any residual benzoyl chloride, cooled, and extracted five times with chloroform; and the combined chloroform extracts were shaken four times with 12–14 c.c. 0·64% HCl. The mixed acid extracts were boiled gently to free them of chloroform, and were then cooled. The benzoylated alkaline solution which had been extracted with chloroform was neutralized, boiled free of chloroform, cooled, and shaken with 2 g. blood charcoal. The charcoal was filtered off, washed with distilled water and eluted by boiling it in 20 c.c. 0·64% HCl, and filtering. The eluate was neutralized to litmus with 20% NaOH and was brought to volume. 5 c.c. of this solution were without pressor activity when injected intravenously into a cat, wt. 2·8 kg., under chloralose anaesthesia.

The cooled acid solution was neutralized with NaOH to a volume of 53 c.c. 0·1 c.c. of this solution was diluted to 10 c.c. with 0·9% NaCl. 1·5 c.c. of this diluted fluid, injected intravenously into a 2·8 kg. cat under chloralose anaesthesia, produced a rise in arterial blood pressure of 40 mm. Hg. The undiluted solution was made alkaline with 5 c.c. 10% NaOH, and was extracted five times with ether. The combined ether extracts, volume approximately 100 c.c., were dried for a week over anhydrous Na_2SO_4. The alkaline solution which had been extracted with ether was neutralized with HCl, boiled to free it of ether, and then shaken with 2 g. blood charcoal. The charcoal was filtered off, eluted by boiling and stirring with 20 c.c. 0·64% HCl, and the whole was filtered. The filtrate after cooling was neutralized to litmus paper with 20% NaOH, and brought to volume. This solution was found to be without pressor activity on intravenous injection, in a dose of 3 c.c., into a cat, wt. 2·8 kg., under chloralose anaesthesia.

After a week the ether was decanted from the Na_2SO_4 which was washed with dry ether, the washings being added to the decanted ether solution. A saturated solution of oxalic acid in ether was added drop by drop until no further turbidity developed. The solution was then spun in closed tubes for an hour. The ether was decanted: it gave no further precipitate on the addition of a few drops of oxalic acid in ether. The precipitate was washed with ether, and crystallized from 15 c.c. absolute alcohol. In 2 hr. a crop of white, warty crystalline groups began to form. The flask was transferred to the ice chest.

The following day the mother liquor was decanted and returned to the ice chest. The crystals, (A1) which had separated, were drained, washed briefly with ice-cold absolute alcohol, again drained and put into a desiccator over soda lime (m.p. 113° C., after drying in air on a porous plate).

No further crystals formed in the mother liquor during the next 12 hr. The solution was taken down gently in a vacuum desiccator to approximately ½ vol., a little solid separating. The flask was then warmed, complete solution taking place. The solution was allowed to cool to room temperature and was then put into the ice chest. Crystals began to separate almost at once (A2). The next day the mother liquor was decanted and allowed to evaporate slowly under a glass jar. The crystals (A2) were drained, shortly washed with ice-cold absolute alcohol, again drained, and put into a desiccator over soda lime. A few of these crystals were dried on a porous plate in air at room temperature, and the melting-point determined. It was 108–109° C. decomposition. The residue from the mother liquor was a slightly yellow waxy mass of ill-formed crystals (A3). These were washed and dried as before.

After 2 days, weighed amounts of each set of crystals were dissolved in 10 c.c. 0·9% NaCl. The weights taken were: A1, 1·27 mg.; A2, 2·77 mg.; A3, 2·1 mg. These solutions were tested for pressor activity by intravenous injection into a cat, wt. 2·3 kg., under chloralose anaesthesia. The results are illustrated in Fig. 1 and are summarized in Table 2. Solutions A 1 and A 2 were diluted 1 in 10 with 0·9% NaCl before injection.

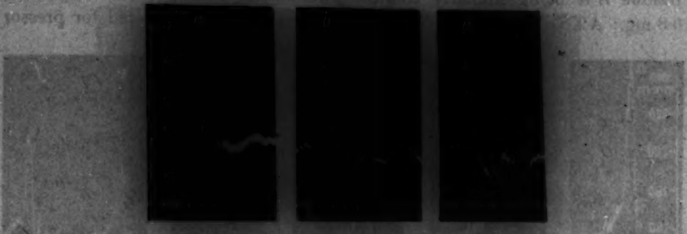


Fig. 1. The tracings show the responses to the following solutions: (*a*) 2 c.c. of a 1/10 dilution of A 1; (*b*) 1 c.c. of a 1/10 dilution of A 2; (*c*) 0·4 c.c. of A 3. Time marker, 10 sec.

TABLE 2

Solution c.c.	Wt. of oxalate μg.	Arterial pressure, mm. Hg		m.p. of crystals °C.
		Base-line	Rise	
1/10 A 1 2·0	25·4	150	39	113
1/10 A 2 1·0	27·7	146	43	102
A 3 0·4	84·0	144	46	83

From these results it was seen that the activity of the A 3 was much lower than that of the A 1 and A 2 crystals.

The A 1 solution was stored in the ice chest. A 1 was at once recrystallized from 3 c.c. absolute alcohol, and next day the crystals (A 4) which had separated were drained, washed with ice-cold alcohol, again drained, and dried over soda lime in a partially evacuated desiccator. The mother liquor from the A 1 recrystallization was allowed to evaporate under a glass jar, and the residual solid dried and reserved as A 4*r*. A 2 was similarly recrystallized, giving crystals A 5, and the mother liquor was used for the recrystallization of A 3, crystals A 6 being obtained. Weighed amounts of these dried crystals were dissolved in 10 c.c. 0·9% NaCl: A 4, 0·45 mg.; A 5, 0·8 mg.; A 6, 1·0 mg. These solutions were tested for pressor activity in a 3·1 kg. cat under chloralose anaesthesia, and comparison was made with the original undiluted solution of A 1 which had been stored in the ice chest for the intervening 9 days. Fig. 2 shows the responses obtained; and these are summarized in Table 3. From these results it was concluded that crystals A 1 had recrystallized, giving crystals A 4 without apparent change in activity, and that crystals A 5 and A 6 were of considerably lower activity than crystals A 1 and A 4.

Recrystallization was repeated. Crystals A 4 were recrystallized from 2 c.c. absolute alcohol, crystals A 7 being obtained. The mother liquor from A 7 was used for the recrystallization of A 5, crystals A 8 being separated, and the mother liquor was used for the recrystallization of A6, crystals A9 being obtained. These crystals were washed, drained, and dried as previously described, and known weights of each were dissolved in 10 c.c. 0·9% NaCl, residue A4*r* being similarly treated. The weights were: A7, 1·5 mg.; A8, 0·8 mg.; A9, 0·35 mg.; A4*r*, 0·6 mg. These solutions were tested for pressor activity in a cat, wt. 2·3 kg. Solution A1 was again used for comparison. The results are illustrated in Fig. 3. 0·15 c.c. of solution A7 containing 22·5 μg., 0·3 c.c. of solution A8 containing 24 μg., 0·4 c.c. of solution A4*r* containing 24 μg., and 0·6 c.c. of solution A9 containing 21 μg., each equates with 0·2 c.c. of solution A1 containing 25·5 μg. From these results it was concluded that crystals A1, A7, A8, A9 and A4*r* all had the same pressor activity.

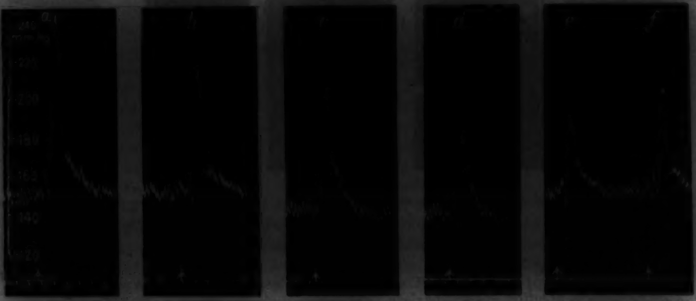


Fig. 2. The tracings show the responses to the following solutions: (*a*) 0·2 c.c. A1; (*b*) 0·1 c.c. A1; (*c*) 0·3 c.c. A5; (*d*) 0·2 c.c. A6; (*e*) 0·2 c.c. A4; (*f*) 0·25 c.c. A4. Time marker, 10 sec.

TABLE 3

Solution c.c.	Wt. of oxalates μg.	Arterial pressure, mm. Hg		m.p. of crystals ° C.
		Base-line	Rise	
A1 0·2	25·4	151	96	113
0·1	12·7	158	64	
A5 0·3	24·0	142	65	107
A6 0·2	20·0	140	44	105
A4 0·2	9·0	150	40	112
0·25	11·5	152	52	

Three melting-point determinations were made on each of these crystal batches, A7 melting at 113° C., A8 at 112° C., A4*r* at 112° C., and A9 at 112° C. The A4 crystals had previously given a m.p. 112° C. In all cases melting was accompanied by decomposition.

Repeated recrystallization was seen to be producing crystals having a pressor activity no greater than that given by crystals A1, the crystals from subsequent recrystallizations having remained of constant pressor activity and melting-point. Moreover, less active crystal batches, which showed a lower melting-point than crystals A 1, reached on recrystallization the pressor activity and melting-point of the original A 1 crystals (crystals A 8 from A 2, and A 9 from A 3). Such crystals were, therefore, judged to be pure.

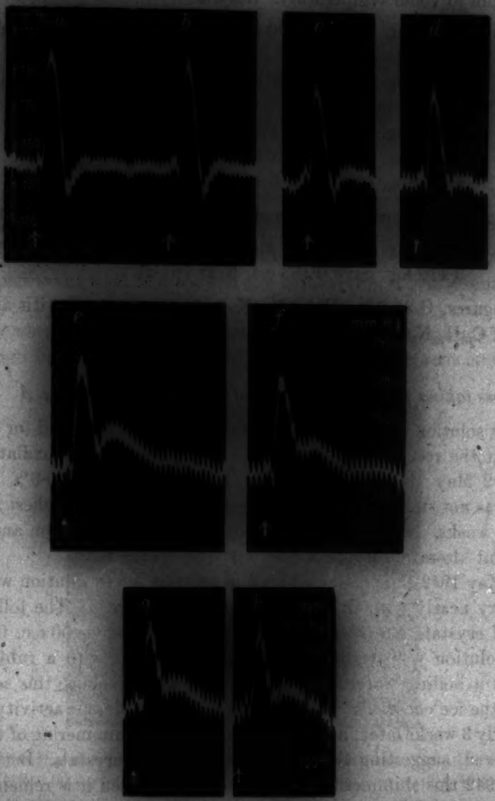


Fig. 3. The tracings show the responses to the following solutions: (*a*) 0·2 c.c. A 1; (*b*) 0·15 c.c. A 7; (*c*) 0·3 c.c. A 8; (*d*) 0·2 c.c. A 1; (*e*) 0·4 c.c. A 4*r*; (*f*) 0·2 c.c. A 1; (*g*) 0·6 c.c. A 9; (*h*) 0·2 c.c. A 1. Time marker, 10 sec.

(*c*) *Analyses and empirical formula of the oxalate of the base A*

Sodium fusion on crystals A 7 showed that the compound contained C, H, N but no halogen, S, or P.

Approximately 22 mg. of crystals A7 were sent immediately to Dr G. Weiler for micro-analysis. Crystals A8 were recrystallized overnight, and approximately 15 mg. were sent next day. The crystals for analysis were dried in a high vacuum at room temperature. Results:

For A7: 3·526 mg. gave 5·800 mg. CO_2 and 1·610 mg. H_2O; residue 0·036 mg.
3·737 mg. gave 6·140 mg. CO_2 and 1·780 mg. H_2O; residue 0·023 mg.
4·398 mg. gave 0·232 c.c. N at 750/21°.
3·833 mg. gave 0·210 c.c. N at 755/19°.
C 44·9%, 44·8%; H 5·1%, 5·3%; N 6·1%, 6·4%.

These figures correspond with an empirical formula of $C_8H_{11}N_1O_6$.

For A8: 3·580 mg. gave 5·980 mg. CO_2 and 1·750 mg. H_2O; no residue.
3·410 mg. gave 0·183 c.c. N at 767/23°.

These figures, C 45·6%, H 5·4%, N 6·3%, correspond with an empirical formula of $C_{18}H_{22}N_2O_{12}$.

(d) *Changes taking place in a solution of the oxalate of the base A*

The first solution of the oxalate crystals A1, which was used for comparison throughout the recrystallizations in the purification of the oxalate, was prepared on 2 May 1942, and contained 1·27 mg. in 10 c.c. 0·9% NaCl. This solution was not sterilized, but was stored, corked, in the ice chest from 2 May 1942 for 5 weeks. No visible change took place in this solution, and there was no apparent alteration in pressor activity (see Table 4).

On 20 May 1942 the pressor activity of crystals A8 in solution was found to equate very nearly with that of crystals A1 (see Fig. 3). The following day, 3·3 mg. of crystals A8 (recrystallized) were dissolved in 50 c.c. 0·9% NaCl, and the solution was sterilized in a bottle provided with a rubber cap, by heating in a boiling water-bath for 30 min. After cooling, this solution was stored in the ice chest. The solution was tested for pressor activity on 9 June 1942, nearly 3 weeks later; and, on shaking, a slight shimmering of the solution was observed, suggesting the separation of minute crystals. Between 9 and 18 June 1942 this shimmering greatly increased. When it is remembered that crystals A8 originally equated with crystals A1, the results given in Table 4 suggest that alteration had occurred in the pressor activity of the solution A8. For the pressor activity tests, the solution was diluted 1:3 with 0·9% NaCl.

On 18 June 1942 examination under the microscope confirmed the presence of minute crystals in the solution. The residual amount of the solution was divided into two parts; one part of the solution was filtered through kieselguhr, and the other part was spun for 45 min., and the supernatant fluid was decanted.

TABLE 4

Solution	Date	Vol. injected c.c.	Wt. of crystals µg.	Arterial pressure, mm. Hg Base-line	Arterial pressure, mm. Hg Rise	Wt. of cat kg.
A1	3. v. 42	0·2	25·4	150	39	2·3
	12. v. 42	0·2	25·4	153	64	3·1
	5. vi. 42	0·2	25·4	113	45	2·5
A8	20. v. 42	0·3	24·0	140	47	2·3 (Fig. 3)
A1	20. v. 42	0·2	25·4	141	46	2·3
A8	9. vi. 42	2·0	44·0	96	60	2·4
	10. vi. 42	4·0	88·0	114	54	2·7
	11. vi. 42	2·0	44·0	138	12	2·9
	12. vi. 42	5·0	122·0	141	42	2·5

Close inspection of these solutions showed that the kieselguhr filtrate was clear, but the supernatant fluid still showed the shimmering of the minute crystals. A cat, wt. 3·4 kg., was prepared as previously described, and 2 c.c. of each of these fluids were injected intravenously without further dilution. The results are illustrated in Fig. 4. During the rise in blood pressure associated with the injection of the supernatant after spinning, there was a clonic fit involving the ears and forelimbs, and lasting about 10 sec.; these fits recurred, always clonic in type. The blood pressure fell to the original base-line in 12 min. Clonic fits recurred with lessening severity during the next 20 min. The oxalate when first isolated was instantly soluble in water. The gradual formation of the insoluble crystals will again be referred to later in this paper (see § 12).

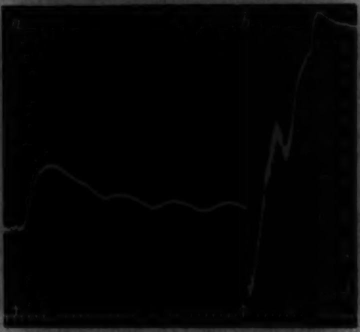


Fig. 4. The tracings show the responses to the following injections: (*a*) 2 c.c. of the filtered solution; (*b*) 2 c.c. of the supernatant after spinning. Time marker, 10 sec.

2. *A repeat isolation of the oxalate of the base A from the steam distillate of urine*

In order to obtain purified material for the preparation of other salts of the base A, and for preliminary examination of the ultra-violet absorption spectrum of this base, the oxalate was again isolated.

The process used in this isolation of the oxalate from 653 l. of the urine of male surgical and convalescent patients was very similar to that employed in the first isolation; progress was less rapid, the time taken extending throughout 13 weeks. Only those points in which this isolation differs from the former are described.

The urine was collected throughout 10 weeks, the charcoal eluates were again worked in groups corresponding to weeks, but were simply concentrated by passing the base from alkaline solution into chloroform, and back into dilute aqueous acid. The chloroform was gently boiled out of these acid aqueous solutions, which were neutralized with NaOH, sterilized by boiling, and were then stored in the ice chest. It was noted after the 5th week that the shimmering appearance described above in solution A8 had begun to appear in the early solutions. A preliminary benzoylation was carried out on the combined solutions corresponding to 2 weeks of urine collection, the base subsequently being passed from alkaline solution to chloroform, and thence to dilute aqueous acid, with concentration to smaller volume. The five aqueous acid solutions resulting were further concentrated by chloroform extraction from alkaline solution, the base being then passed back to a small volume of dilute HCl. The chloroform was gently boiled out of this solution, which was then cooled and neutralized; solid NaOH was gradually added to 10% with cooling and stirring, and benzoylation was repeated. The base was again passed into chloroform, and thence into dilute aqueous acid. After being boiled to remove all traces of chloroform, cooled, and made alkaline with NaOH, the solution was thoroughly extracted with ether, the ether extracts being dried over anhydrous Na_2SO_4 for a week. The oxalate was precipitated in the manner previously described, and after it had been washed with ether, was recrystallized from absolute alcohol. The melting-point of these crystals was 97° C. After they had been recrystallized twice from absolute alcohol, and dried in a high vacuum at room temperature, their melting-point was 112° C.

(a) *Analysis of the oxalate crystals*

Approximately 18 mg. of these crystals were sent (17 August 1942) for micro-analysis. The crystals were dried in a high vacuum at room temperature before analysis:

3·919 mg. gave 6·500 mg. CO_2, and 1·990 mg. H_2O.
3·912 mg. gave 6·520 mg. CO_2, and 1·950 mg. H_2O.
3·620 mg. gave 0·217 c.c. N at 757/21°.
3·680 mg. gave 0·218 c.c. N at 757/22°.
C 45·26%, 44·4%; H 5·64%, 5·54%; N 6·9%, 6·8%.

These figures for C and H agree fairly well with those of the previous analyses. The N figures are rather higher for the second crystals.

These oxalate crystals were, however, of low activity. Minute needle crystals began to separate in 24 hr. from their solution in 0·9% NaCl. On 17 August 1942 1·2 mg. of the oxalate were dissolved in 10 c.c. 0·9% NaCl, and were tested for pressor activity on a cat, wt. 1·8 kg., under chloralose anaesthesia.

0·6 c.c. of this solution containing 72 μg. were required to raise the mean arterial pressure 40 mm. Hg from a base-line of 104 mm. Hg; this response equated roughly with 10 μg. of adrenaline tartrate. The remainder of these oxalate crystals was used for the preparation of the free base A, of the aurichloride and of the platinichloride, and for examination of the ultra-violet absorption of the base A in solution as the hydrochloride.

(b) *The free base A, and the determination of its molecular weight*

The free base A, an oil, was isolated from the oxalate, and the molecular weight was determined by means of the depression of the freezing-point of camphor.

Approximately 9 mg. oxalate crystals dissolved in 10 c.c. water were made alkaline with NaOH, and extracted with ether. The ether extracts were dried for 4 days over three changes of anhydrous Na_2SO_4, and the ether solution was decanted. The ether was distilled off, and the remaining colourless oil was dried in a high vacuum at 60° C. for several hours, the oil showing some volatility under these conditions. In 24 hr. the oil became slightly yellow. In an attempted microboiling-point determination, above 150° C. the oil became dark yellow, by 210° C. it was dark reddish brown: the boiling-point was not reached by 220° C. at atmospheric pressure.

The oil was sent for a molecular weight determination:

0·312 mg. oil in 3·380 mg. camphor gave a depression of 20° C.; mol. wt. 171.

(c) *The aurichloride of the base A*

A solution of approximately 4·5 mg. oxalate crystals in 10 c.c. water was made alkaline with NaOH, and extracted with ether. The ether extracts were washed with water, and then shaken with 2 c.c. 1% HCl. The aqueous acid solution was warmed to free it from ether, and then treated with a drop of a concentrated solution of aurichloride in 2% HCl. Crystals separated in 4 days. They were drained, rapidly washed with ice-cold water, and dried. The crystals were yellow, rather soluble, and needle shaped. After drying in a high vacuum at room temperature, they darkened at 150–155° C. and melted with decomposition at 183–185° C., or higher, depending on the rate of heating.

(d) *The platinichloride of the base A*

The platinichloride was prepared from 12 mg. oxalate crystals; the base was passed from alkaline solution to ether and back to 3 c.c. 5% HCl. The aqueous phase was boiled to dispel ether, and a few drops of chloroplatinic acid solution were added. No crystals separated in 2 days. The solution was concentrated to approximately ½ vol.; crystals did not separate. The addition of 1 vol. of alcohol was followed by the rapid formation of orange-yellow crystals, which were filtered off, washed thoroughly with ice-cold alcohol, then with ether, and dried in air. They were rather soluble in water, but almost insoluble in alcohol.

The crystals after drying in air darkened at 250–255° C., and melted with decomposition at about 275–270° C. Approximately 16 mg. of these crystals were sent for micro-analysis. They were dried in a high vacuum at 100° C. before analysis:

4·075 mg. gave 3·200 mg. CO_2, 3·134 mg. H_2O, and 1·380 mg. residue.
3·776 mg. gave 2·460 mg. CO_2, 1·100 mg. H_2O, and 1·289 mg. residue.
3·775 mg. gave 0·150 c.c. N at 750/24°.
3·610 mg. gave 0·137 c.c. N at 750/23°.
C 21·45%, 21·4%; H 3·134%, 3·26%; Pt 33·87%, 34·11%; N 4·5%, 4·31%.

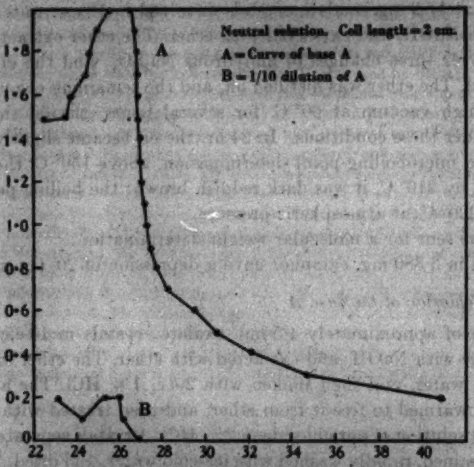


Fig. 5. The curves show the ultra-violet absorption of the base A obtained from the steam distillate of alkaline urine concentrate. Ordinates: density. Abscissae: λ in A. × 10^{-2}

(e) *The precipitation of the base A by $HgCl_2$*

The base A has been shown to be precipitated from urine and urine concentrate by $HgCl_2$ [Lockett, 1944]. By working up the final mother liquor from the last recrystallization of the oxalate described above, a further 11 mg. of oxalate crystals, m.p. 112° C., were obtained. A solution of these crystals (11 mg.) in 5 c.c. water was made acid with HCl and thoroughly extracted with ether. After separation, the ether was boiled out of the aqueous phase, which was then cooled, and exactly neutralized with NaOH; one drop of conc. HCl was then added, and the volume was brought to 12 c.c. 2 c.c. of a cold saturated solution of $HgCl_2$ were then added, and greyish white crystals separated within 24 hr. These crystals were completely insoluble in cold water and in alcohol, and failed to recrystallize from hot water.

(*f*) *The ultra-violet absorption spectrum of the base A*

Eluates from blood charcoal adsorption columns which contained the base A were found to show an absorption band in the ultra-violet at approximately 2600 A. [Lockett, 1944]. The base A was therefore examined in solution as the hydrochloride, and was found to give a similar absorption spectrum.

0·6 mg. of the oxalate crystals, the analysis of which is given above, were dissolved in 5 c.c. water. 1 c.c. 5% NaOH (A.R.) was added, and the solution was thoroughly extracted with ether. The ether extracts were combined, and were shaken with 5 c.c. 1% HCl. The acid-aqueous phase, warmed in a water-bath at 60° C. for 20 min. to remove all traces of ether, was neutralized with 0·65 c.c. 10% NaOH (A.R.) and brought to a volume of 6 c.c. A control solution of similar NaCl content was prepared. Ultra-violet absorption spectra of this solution and of a 1/10 dilution were photographed. The results are illustrated in Fig. 5 and show the presence of an absorption band at 2600 A.

3. *An investigation of the function of the oxygen and nitrogen contained in the empirical formula of the oxalate of the base A*

The empirical formula of the oxalate of the base A isolated by the method of steam distillation is $C_{16}H_{19-22}N_2O_{5\cdot 5-6}$. It had been observed that the pressor activity of the oxalate solutions decreased with time. In order to study the chemical nature of the N and O in as active a form of the base A as possible, and to avoid the necessity of working up the enormous volumes of urine which would be required to furnish enough of the pure compound for a single conclusive chemical test, the following experiments were carried out. The facts that the base A was so readily and completely extracted from aqueous alkaline solution by ether, and that it passed back quantitatively from the ether to dilute aqueous acid, rendered possible the method of working described below.

A total of 120 l. of urine were concentrated, cooled, filtered and steam distilled exactly as was described in detail in the previous section of this paper. The steam distillate was similarly treated with charcoal, and the charcoal eluates were worked up exactly as was described for a single group during the isolation of the base A, as far as the first benzoylation. Care was taken that this benzoylation should be complete. It was continued until no further separation of a precipitate occurred on re-benzoylation of the supernatant after spinning: the removal of the base B must therefore have been complete.

The supernatant solution was warmed to destroy any remaining benzoyl chloride, cooled, and then thoroughly extracted with chloroform. From the chloroform the base was passed back into 50 c.c. 1% HCl. The acid solution was boiled gently to free it of chloroform, and after cooling was neutralized with NaOH, and diluted with water to 130 c.c. This dilution was called solution A, and served for the preparation of the solutions described below.

All ether extractions were carried out in the following manner. The solution was transferred to an extraction funnel (20 c.c.) and washed in with water to bring the volume to 8 c.c. 0·3 c.c. 10% NaOH were added, and three extractions each with 6 c.c. ether were made, shaking for 5 min. each time. The combined ether extracts were shaken for 3 min. with 10 c.c. 0·64% HCl, and the aqueous phase was separated. When all the solutions had been prepared to this stage, the tubes were heated together in a water-bath at 86–90° C. for 10 min. to free them from ether. After cooling, 1 c.c. 10% NaOH was added to each tube, the solution was neutralized with 5% HCl, and made up to 15 c.c. with water. As the solutions were prepared in the course of 3 days, a control extraction was carried out on 5 c.c. of solution A on each of these days, and the extractions were numbered in sequence C*a*, C*b*, C*c*. Where there was any divergence in treatment a separate control was prepared, and was numbered according to the experiment tube, but with the suffix -*c*.

Tube 1. 5 c.c. solution A were treated with a concentrated aqueous solution of $HgCl_2$, 1 c.c. After standing 24 hr. the solution was filtered through kieselguhr, saturated with H_2S, filtered, and neutralized. The solution was then made alkaline and extracted with ether, etc., as described above.

Tube 2. 5 c.c. solution A were treated with 1 c.c. of a saturated solution of $NaHSO_3$. After the tube had stood 24 hr. a fine opalescence was seen, which failed to filter. The solution was made alkaline and extracted with ether as described above.

Tube 3. 5 c.c. solution A were treated with 0·3 c.c. 10% NaOH and were extracted with ether, but the ether extracts were treated in a manner different from that described above. The combined ether extracts were washed for 30 sec. with 5 c.c. of a solution containing 0·5 c.c. 5% $NaHSO_3$ and 4·5 c.c. phosphate buffer, *p*H 7·4. After separating, the ether extract was shaken with 0·64% HCl as previously described.

Tube 3 *c*. 5 c.c. solution A were treated in a way similar to that described for tube 3, but the ether extracts were washed with 4·5 c.c. of the buffer brought to 5 c.c. with water.

Tube 4. 5 c.c. solution A were treated with 1 drop of conc. HCl, and 1 c.c. of a saturated aqueous solution of 2, 4-dinitrophenylhydrazine. After the solution had stood 30 hr., it was filtered through kieselguhr, neutralized, and was then treated as previously described.

Tube 5. 5 c.c. solution A, 2 drops glacial acetic acid, and 1 c.c. of a concentrated solution of semicarbazide were saturated with solid sodium acetate; after 36 hr. the solution was filtered, neutralized, and then treated as described above.

Tube 6. 5 c.c. solution A were treated with 1 c.c. Nessler's reagent. A yellow crystalline precipitate formed. After standing 24 hr., the solution was filtered, and the filtrate was extracted with ether without the further addition of alkali.

Tube 7. 5 c.c. solution A were saturated with $KClO_4$. After standing 48 hr. the solution was filtered, made alkaline, and extracted in the standard manner.

Tube 8. 5 c.c. solution A were saturated with sodium thiosulphate, and after standing 48 hr. the solution was filtered, made alkaline, and extracted in the standard manner.

Tube 9. 5 c.c. solution A were treated with 1 c.c. freshly prepared sodium hypobromite solution. After the solution had stood 24 hr. the ether extraction was carried out, but no further alkali was added.

Tube 9 *c*. 5 c.c. solution A were treated with 1 c.c. 40% NaOH, and after standing 24 hr. were extracted with ether without the further addition of alkali.

Tube 10. 5 c.c. solution A were treated with 0·5 g. $NaNO_2$, and 0·1 c.c. 5% HCl were added 2-hourly to a total of 1 c.c. No reaction was observed. After 12 hr. the solution was neutralized and extracted in the standard way.

Tube 11. Set up and worked exactly like tube 10, but $NaHSO_3$ was substituted for the nitrite.

Tube 12. 5 c.c. solution A plus 0·1 g. NaOH, were cooled in ice, and a Schotten-Baumann benzoylation was carried on for 2 hr., 1 drop 40% NaOH being added as required. No reaction was apparent. A duplicate tube in which normal saline was substituted for solution A was worked in parallel, and at the end of the 2 hr. was titrated with 3·65% HCl. 2·1 c.c. of the acid were required to neutralize the solution to cresol red. From this was calculated the amount of alkali in the experiment tube. This tube was warmed on a water-bath at 40° C. for 10 min., then neutralized, spun and decanted. The decanted fluid was extracted in the standard way.

Tube 12 *c*. 5 c.c. solution A plus 0·1 g. NaOH were stood throughout the benzoylation in ice-water. At the end of the 2 hr., 0·4 c.c. 3·65% HCl were added to adjust the strength of alkali in the control tube to that in the experiment tube before heating in alkaline solution. The tube was now worked up exactly as described for the experiment tube.

Tube 13. 5 c.c. solution A were treated exactly as described for tube 3, but the ether extracts were washed for 60 sec. with 5 c.c. of a concentrated solution of semicarbazide hydrochloride saturated with sodium acetate.

Tube 13 *c*. 5 c.c. solution A were treated as tube 13, but the ether extracts were washed with 5 c.c. of a saturated solution of sodium acetate.

Tube 14. Identical with tube 13 except that the ether extracts were washed with an aqueous solution of 2, 4-dinitrophenylhydrazine hydrochloride, 5 c.c.

Tube 14 *c*. As tube 14, but the ether extracts were washed with 5 c.c. water.

The final solutions, numbered in correspondence with the tubes, were now tested for pressor activity on a cat, wt. 2·9 kg., and prepared in the manner previously described. The responses obtained on intravenous injection of the test solutions are shown in Fig. 6.

The control solutions C*a*, C*b*, C*c*, made on three consecutive days, equated very closely; there was therefore no appreciable alteration in the activity of solution A during this period. Between injections 21 and 22 the cat was rested for 88 min., and thereafter there was increasing response to the same dose of active solution. This is shown by the responses 23 and 28 to 0·3 c.c. of solution 13*c*, and by comparison of responses 4 and 11 on the one hand, with response 29 on the other, 0·3 c.c. of control solution being injected on each of these three occasions. No. 2 is the response to 1·0 c.c. of solution 1; complete destruction of activity has been produced by $HgCl_2$. No. 3 is the response to 0·3 c.c. of solution 2. The slight reduction in the activity of this solution would be without significance were it not for the responses seen in nos. 26 and 27, which resulted from the injection of 0·3 c.c. of solutions 3 and 3*c*. These responses suggest the possibility of the presence of a carbonyl group. No. 9 shows the response to 0·3 c.c. of solution 4; there has been no separation of a dinitrophenylhydrazone. When the ether extract washing method was employed, however, a reduction in activity occurred, as is shown by comparison of the responses to 0·3 c.c. of solution 14 (no. 24) and 0·3 c.c. of solution 14*c* (no. 25). No. 10 shows the response to 0·3 c.c. of solution 5, and comparison of this with the response to the control solution (no. 4) shows that a slight reduction in activity has occurred: when the ether extract washing method was used, there was total loss of pressor activity, no. 22 showing that there is no response to 0·3 c.c. of solution 13. This was confirmed by a further injection of solution 13 between nos. 27 and 28. Nos. 23 and 28 are the responses to 0·3 c.c of solution 13*c*. Again the possible presence of a carbonyl group is indicated.

No. 12 shows the response to 1 c.c. of solution 6; there has been precipitation of the base by Nessler's reagent, as is seen by comparison with no. 11, which is the response to 0·3 c.c. of the control. No. 13 is the response to 0·3 c.c. of solution 7: the slight reduction of activity cannot be considered of significance until confirmed by more vigorous oxidation. No. 15 shows the response to 0·3 c.c. of solution 9. It equates exactly with 0·3 c.c. of its control 9c (no. 14). The base A is, then, unaffected by sodium hypobromite. No. 16 shows the

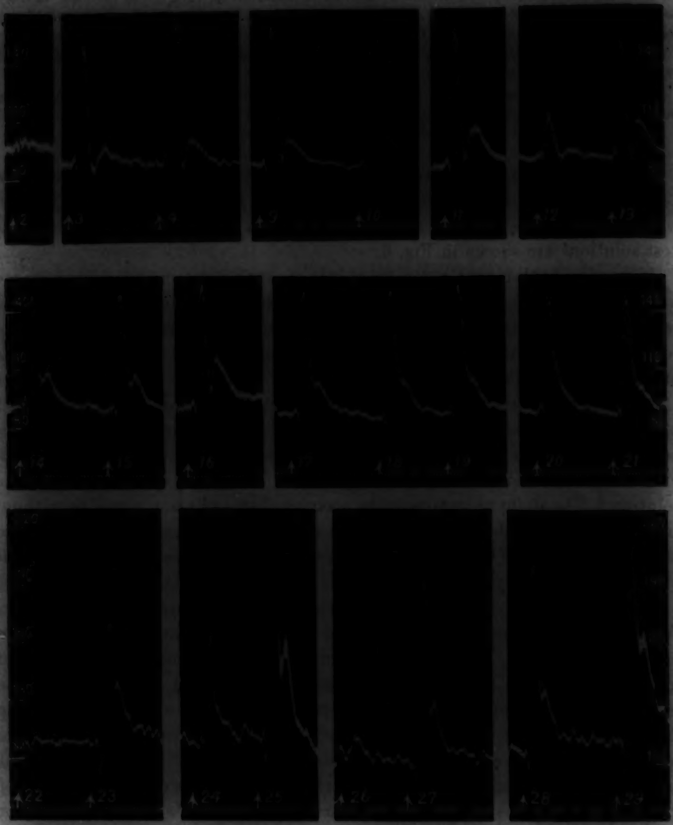
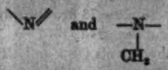


Fig. 6. The tracings show the responses to the following solutions: (2) 1·0 c.c. soln. 1; (3) 0·2 c.c. soln. 2; (4) 0·3 c.c. control; (9) 0·3 c.c. soln. 4; (10) 0·3 c.c. soln. 5; (11) 0·3 c.c. control; (12) 1·0 c.c. soln. 6; (13) 0·3 c.c. soln. 7; (14) 0·3 c.c. soln. 9; (15) 0·3 c.c. soln. 9 *c*; (16) 0·3 c.c. soln. 8; (17) 0·3 c.c. soln. 10; (18) 0·3 c.c. soln. 11; (19) 0·3 c.c. control; (20) 0·3 c.c. soln. 12; (21) 0·3 c.c. soln. 12 *c*; (22) 0·2 c.c. soln. 13; (23) 0·3 c.c. soln. 13 *c*; (24) 0·3 c.c. soln. 14; (25) 0·3 c.c. soln. 14 *c*; (26) 0·3 c.c. soln. 3; (27) 0·3 c.c. soln. 3 *c*; (28) 0·3 c.c. soln. 13 *c*; (29) 0·3 c.c. control. Between injections 21 and 22 the cat was rested for 88 min. B.P. scale in mm. Hg. Time marker, 5 sec.

response to 0·3 c.c. of solution 8, and it equates well with that to 0·3 c.c. of the control (no. 19), thus showing that treatment of the base with sodium thiosulphate has had no effect. No. 17 shows the response to 0·3 c.c. of solution 10. It equates with that to 0·3 c.c. of its control solution (no. 19), thus showing that the base A is unaffected by nitrite in acid solution. No. 18 shows the response to 0·3 c.c. of solution 11; this equates with that to the control solution (no. 19). Nos. 20 and 21 show that the response to 0·3 c.c. of solution 12 equates with that to 0·3 c.c. of the control (no. 12*c*). This again demonstrates the failure of the base A to benzoylate, and in addition serves as a control to solution A, confirming that the pressor response being recorded is solely that of the base A. The responses nos. 22–29 have been discussed above. The results of this experiment show that the base A contains no primary or secondary amino-N, and indicate the possible presence of a carbonyl group.

A second experiment was designed to confirm the tertiary nature of any basic N in the base A, and to study further the O present in the empirical formula. Were the base A a tertiary amine, an amine oxide, if obtained, would probably prove to be insoluble in ether. A distinction might be made between



$$\diagdown N \diagup \quad \text{and} \quad \underset{CH_3}{\underset{|}{-N-}}$$

by the removal of an alkyl group and subsequent benzoylation. Were the excess O atoms present in the empirical formula of the oxalate not associated with alcohol or water of crystallization, their function had yet to be determined. The stability of the base to strong alkali and mineral acid at room temperature was therefore studied, and an attempt was made to exclude the presence of certain diketones.

The remaining volumes of the control solutions C*a*, C*b* and C*c* from the previous experiment were combined, and formed solution B.

All benzoylations were carried out in the same manner. The solution was neutralized to litmus paper, and 0·1 g. NaOH was then added, and dissolved; benzoyl chloride was added, 1 drop every 10 min., vigorous shaking being maintained. Small amounts of powdered NaOH were added as required. All solutions to be benzoylated were worked up in parallel, and the process was continued for 3 hr. The tubes were then warmed in a water-bath at 40° C. for 20 min. The solutions were spun, the supernatant was decanted, neutralized to litmus, spun again, and the supernatant decanted, made alkaline and extracted with ether.

All ether extractions were carried out as described in the last experiment. The combined ether extracts from each solution were shaken with 10 c.c. 0·7 % HCl, the ether being removed from the aqueous acid solution, after separating, by standing the tubes in a water-bath at 90° C. for 10 min. The solutions were then neutralized with NaOH, and were brought to a volume of 12 c.c. with water.

Tubes 1–6 were put up at the same time, left standing for 75 hr., and then treated as described below:

Tube 1. Solution B 1 c.c., water 3 c.c., 50% H_2SO_4 1 c.c. (10% H_2SO_4).
Tube 2. Solution B 1 c.c., water 2 c.c., 50% H_2SO_4 2 c.c. (20% H_2SO_4).
Tube 3. Solution B 1 c.c., water 1 c.c., 50% H_2SO_4 3 c.c. (30% H_2SO_4).
Tube 4. Solution B 1 c.c., water 4 c.c., NaOH solid 0·5 g. (10% NaOH).
Tube 5. Solution B 1 c.c., water 4 c.c., NaOH solid 1·0 g. (20% NaOH).
Tube 6. Solution B 1 c.c., water 4 c.c., NaOH solid 1·5 g. (30% NaOH).

These solutions were neutralized to cresol red as internal indicator by the addition of conc. H_2SO_4 drop by drop, or of powdered NaOH, in each case with cooling. The volume of each was made up to 8 c.c., and the solution made alkaline and extracted with ether, etc., in the way already described. A control tube (tube C1) containing 1 c.c. solution B to which 4 c.c. water had been added stood throughout this period, and was then made alkaline and extracted with ether along with the tubes 1–6.

Tube 7. 1 c.c. solution B was treated with 4 c.c. freshly prepared chlorine water, and one drop conc. HCl was added. After standing 62 hr. at room temperature, the solution was neutralized, benzoylated, and extracted, etc., in the standard way.

Tube 8. 1 c.c. solution B was treated with 4 c.c. 10 vol. H_2O_2 solution which had been neutralized with $MgCO_3$. After standing 75 hr., the solution was made alkaline and extracted, etc., in the standard way.

Tube 9. 1 c.c. solution B was treated with 4 c.c. of a dark pink solution of $KMnO_4$ which contained 2 drops conc. H_2SO_4. After standing 73 hr., the solution was neutralized, made alkaline, and extracted, etc. Some reduction had occurred.

Tubes 10–15 and the corresponding controls CB1 and CB2 were set up together, allowed to stand 68 hr., and then worked up.

Tube 10. 1 c.c. solution B was treated with 1 c.c. 10% KI, and 1 drop conc. HCl.
Tube 11. 1 c.c. solution B was treated with 4 c.c. freshly prepared bromine water.
Tube 12. To 1 c.c. solution B 1 c.c. 10% NaOH was added, and 1 c.c. of a saturated solution of Na_2SO_3.
Tube 13. 1 c.c. solution B was treated with 2 c.c. 10% NaOH and zinc dust.
Tube 15. 1 c.c. solution B was treated with 1 c.c. of a saturated solution of phenol.
Tube CB1. 1 c.c. solution B was treated with 1 c.c. 10% NaOH.
Tube CB2. 1 c.c. solution B was treated with 4 c.c. water.

These solutions were neutralized after standing, and were brought to a volume of 8 c.c. They were then benzoylated and extracted with ether, etc., in the manner previously described. Control ether extractions on solution B were made on each of the 3 days during which these solutions were being prepared—*Ca*, *Cb*, *Cc*.

These solutions were now tested for pressor activity on a cat, wt. 1·8 kg. Fig. 7 shows the responses obtained. No. 1 is the response to 4 c.c. of the control solution CB2. No. 2 shows complete loss of pressor activity in 4 c.c. of solution 7. This loss of activity might have been due to the removal of an alkyl group from the N with the production of a secondary amine which benzoylated, but a variety of other reactions are possible (see nos. 11, 12 and 4). No. 3 is the response to 4 c.c. of solution 10. The base A is still present after treatment with KI in acid solution. No. 4 shows complete loss of pressor activity in 4 c.c. of solution 11. Bromine water has reacted with the base A. No. 5 shows the response to 4 c.c. of CB2. No. 6 shows the response to 4 c.c. of solution 12, no. 7 that to 4 c.c. of solution 13, no. 8 that to 4 c.c. of CB1, and no. 9 shows the response to 4 c.c. of solution 15. No evidence of quinone was obtained.

No. 10 shows the response to 3 c.c. of *Ca*, and no. 11 the response to 3 c.c. of solution 8. Treatment with H_2O_2 has lessened the activity. No. 12 is the response to 3 c.c. of solution 9: there is total loss of activity. The formation of an amine oxide is but one possible explanation of these results. No. 13 shows the response to 3 c.c. of *Cc*, no. 14 that to 3 c.c. of solution 1, no. 15 that to 3 c.c. of solution 4, no. 16 to 3 c.c. of solution 3, no. 17 to 3 c.c. of solution 5, no. 18 to 3 c.c. of solution 3, no. 19 to 3 c.c. of solution 6. The base A is therefore stable at room temperature for 3 days to 10 and 20% NaOH or H_2SO_4,

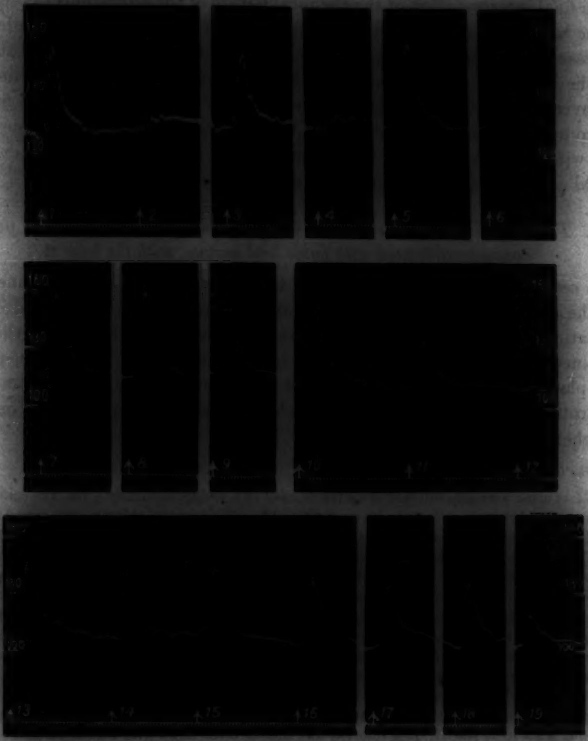


Fig. 7. The tracings show the responses to the following solutions: (1) 4 c.c. soln. CB2; (2) 4 c.c. soln. 7; (3) 4 c.c. soln. 10; (4) 4 c.c. soln. 11; (5) 4 c.c. soln. CB2; (6) 4 c.c. soln. 12; (7) 4 c.c. soln. 13; (8) 4 c.c. soln. CB1; (9) 4 c.c. soln. 15; (10) 3 c.c. soln. *Ca*; (11) 3 c.c. soln. 8; (12) 3 c.c. soln. 9; (13) 3 c.c. soln. *Cc*; (14) 3 c.c. soln. 1; (15) 3 c.c. soln. 4; (16) 3 c.c. soln. 2; (17) 3 c.c. soln. 5; (18) 3 c.c. soln. 3; (19) 3 c.c. soln. 6. B.P. scale in mm. Hg. Time marker, 5 sec.

stable to 30% H_2SO_4, but unstable to 30% NaOH. A control saline injection of 3 c.c. was without effect on the blood pressure, and the responses to *Ca*, *Cb*, *Cc* equated very closely.

No final interpretation of the chemical nature of the alterations taking place in the base A associated with reduction or loss of activity could be made, since the reaction products were not isolated, but any structural formula postulated for the base must be compatible with these findings. Strong indication has, however, been obtained of the tertiary nature of any basic N, and the findings are compatible both with the ability of the compound to form an amine oxide, and with the possibility of the presence of an N-alkyl group. The evidence would also suggest the presence of a carbonyl group.

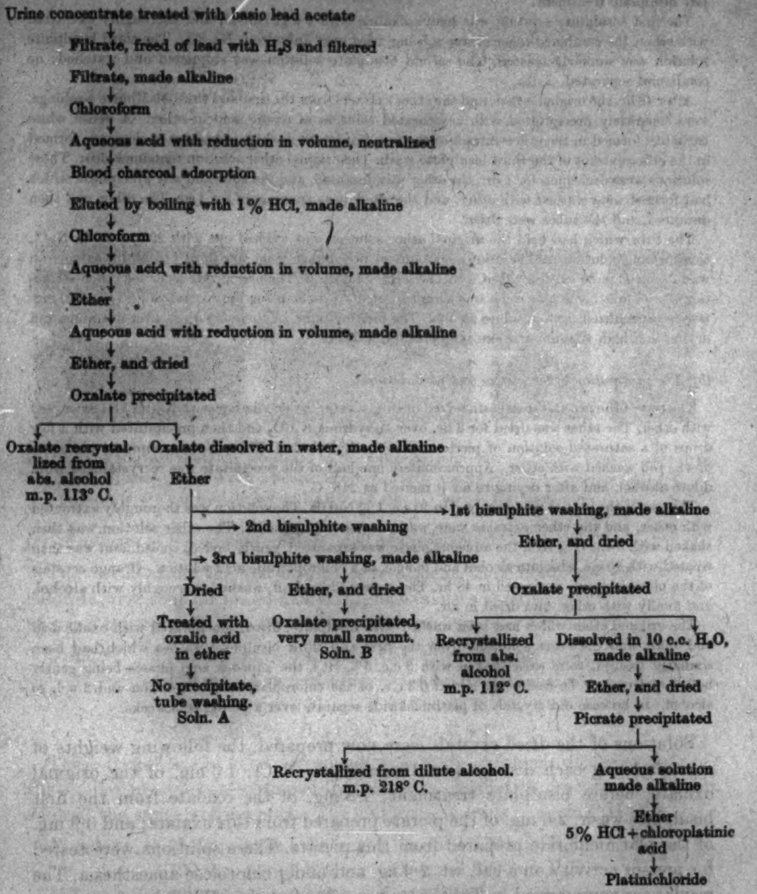
4. *The identification of the pressor base, obtained from the filtrate of urine concentrate treated with basic lead acetate, with the base A*

In order to establish the identity of the similar pressor bases obtained first by the method of steam distillation, and second from the filtrate after precipitating urine concentrate with basic lead acetate, sufficient of the base obtained by the second method was isolated for analysis. The oxalate, picrate and platinichloride were prepared, and a study was made of the ultra-violet absorption spectrum. The method by which the salts were prepared is outlined in Scheme III.

(*a*) *The isolation of this base as the oxalate*

108 l. of urine were concentrated in six fractions of 18 l. to approximately $\frac{1}{8}$ vol. The concentrate was cooled, and then precipitated with an unfiltered basic lead acetate solution (300 g. lead acetate, 150 g. litharge, in 1 l. of water), 1 l. of this solution being added to the concentrate from 18 l. of urine. After the mixture had stood 36–48 hr. the precipitate was removed by filtration, and the filtrate freed from lead by means of H_2S. The PbS was filtered off, and the filtrate was reconcentrated. The combined final concentrates had a volume of approximately 4 l. This solution was made weakly alkaline to litmus by the addition of 20% NaOH and was then filtered through kieselguhr. The filtrate was divided into four parts, each of approximately 1 l., and each part was extracted four times by mechanical shaking with 200 c.c. chloroform. All the chloroform extracts were combined, the volume being 3,160 c.c. The chloroform, in three fractions each of approximately 1 l., was shaken three times with 100 c.c. vol. of 1·0% HCl, all the aqueous acid extracts from the three chloroform fractions being then combined, boiled, cooled, and neutralized to litmus with 10% NaOH. This solution was shaken three times with 3 g. blood charcoal, the charcoal being removed by filtration. The 9 g. charcoal were eluted by boiling and stirring with 300 c.c. 1% HCl. The eluate was filtered, cooled and neutralized with NaOH. After standing to allow the precipitate to flocculate, it was filtered and made alkaline with NaOH, and then thoroughly extracted four times with 50 c.c. chloroform. The combined chloroform extracts were shaken four times with 30 c.c. 1% HCl. The acid extracts were boiled, cooled, and made alkaline with NaOH, and were extracted three times with ether. The combined ether extracts were shaken three times with 20 c.c. 0·64% HCl. The acid aqueous solution was boiled, cooled, and neutralized. A qualitative colorimetric test showed absence of the base B in 0·5 c.c. of this solution [Lockett, 1944]. The solution was made alkaline with NaOH, and was extracted four times with ether. The combined ether extracts, volume approximately 100 c.c., were dried for 48 hr. over anhydrous Na_2SO_4; the ether was then decanted and was treated with a saturated solution of oxalic acid in ether. A dense white turbidity resulted. The precipitate was spun down and washed with ether.

SCHEME III


Urine concentrate treated with basic lead acetate
Filtrate, freed of lead with H_2S and filtered
Filtrate, made alkaline
Chloroform
Aqueous acid with reduction in volume, neutralized
Blood charcoal adsorption
Eluted by boiling with 1% HCl, made alkaline
Chloroform
Aqueous acid with reduction in volume, made alkaline
Ether
Aqueous acid with reduction in volume, made alkaline
Ether, and dried
Oxalate precipitated
Oxalate recrystallized from abs. alcohol m.p. 113° C.
Oxalate dissolved in water, made alkaline
Ether
1st bisulphite washing, made alkaline
2nd bisulphite washing
3rd bisulphite washing, made alkaline
Dried
Treated with oxalic acid in ether
No precipitate, tube washing. Soln. A
Ether, and dried
Oxalate precipitated, very small amount. Soln. B
Ether, and dried
Oxalate precipitated
Recrystallized from abs. alcohol m.p. 112° C.
Dissolved in 10 c.c. H_2O, made alkaline
Ether, and dried
Picrate precipitated
Recrystallized from dilute alcohol. m.p. 218° C.
Aqueous solution made alkaline
Ether
5% HCl + chloroplatinic acid
Platinichloride

Approximately nine-tenths of the precipitate were dissolved in 25 c.c. water, and the remaining small fraction, approximately 3 mg., recrystallized from 0·5 c.c. absolute alcohol. The crystals were drained, and then dried in a high vacuum at room temperature. The m.p. was 113° C.; yield 1·6 mg.

The aqueous solution of the oxalate was made alkaline by the addition of 0·3 g. NaOH and was thoroughly extracted four times with ether. The ether extracts were combined, and were shaken for 15 sec. with 20 c.c. 1·0% sodium bisulphite: this was repeated twice, the three bisulphite solutions being kept separately. The ether solution was dried over anhydrous Na_2SO_4 after the last bisulphite treatment.

The first bisulphite solution was made alkaline with 0·2 g. NaOH, and thoroughly extracted with ether, the combined ether extracts being dried over anhydrous Na_2SO_4. The third bisulphite solution was similarly treated. The second bisulphite solution was stoppered and watched: no compound separated.

After 48 hr. the original ether, and the ether extracts from the first and third bisulphite washings, were separately precipitated with a saturated solution of oxalic acid in ether. A dense white turbidity formed in the ether extract of the first bisulphite wash, a very faint opalescence formed in the ether extract of the third bisulphite wash. The original ether solution remained clear. These solutions were each spun for 1 hr., the ether was decanted, and the tube and any precipitate which had formed were washed with ether, and the whole was spun again for 1 hr. The ether was then decanted, and the tubes were dried.

The tube which had held the original ether solution was washed out with 20 c.c. 0·9% NaCl, this forming solution A. The minute amount of precipitate from the third bisulphite extraction was dissolved in 20 c.c. 0·9% NaCl, this forming solution B. The precipitate from the ether extraction of the first bisulphite wash was scraped out, its weight being approximately 50 mg. 20 mg. were recrystallized from absolute alcohol. The melting-point of these crystals, after draining, and drying in a high vacuum at room temperature, was 112° C.

(b) *The preparation of the picrate and platinichloride*

The rest of the precipitate was dissolved in 10 c.c. water, made alkaline with NaOH and extracted with ether. The ether was dried for 2 hr. over anhydrous K_2CO_3 and then precipitated with a few drops of a saturated solution of picric acid in methyl alcohol. The yellow precipitate was spun down, and washed with ether. Approximately one-half of the precipitate was recrystallized from dilute alcohol, and after drying in air it melted at 218° C.

The rest of the picrate was dissolved in 10 c.c. 1% NaOH. The solution was thoroughly extracted with ether, and the ether extracts were washed with 0·1% NaOH. The ether solution was then shaken with 5 c.c. 5% HCl, the aqueous phase was separated, gently boiled, cooled, and was then treated with 10 c.c. absolute alcohol and 0·5 c.c. of a chloroplatinic acid solution. Orange crystals of the platinichloride separated in 48 hr. These were filtered off, washed thoroughly with alcohol, and finally with ether, and dried in air.

The original ether which had been washed with bisulphite, dried, precipitated with oxalic acid and spun, and the ether solutions from the first and third bisulphite washes which had been similarly treated, were each shaken with 3 c.c. 5% HCl, the aqueous acid phases being gently boiled and cooled. To each were added 0·3 c.c. of the chloroplatinic acid solution and 2 vol. of alcohol. In no case did crystals of platinichloride separate over a period of 3 weeks.

Solutions of the dried crystals were now prepared, the following weights of crystals being each dissolved in 10 c.c. 0·9% NaCl: 1·3 mg. of the original oxalate, before bisulphite treatment; 1·8 mg. of the oxalate from the first bisulphite wash; 2·0 mg. of the picrate prepared from this oxalate; and 0·9 mg. of the platinichloride prepared from this picrate. These solutions were tested for pressor activity on a cat, wt. 2·6 kg. and under chlorolose anaesthesia. The results are summarized in Table 5 and are illustrated in Fig. 8.

From these results it was seen that no further purification of the oxalate occurred as a result of bisulphite washing of its ether solution and subsequent reprecipitation. The pressor response to 80 μg. of the picrate equated approximately with that to 65 μg. of the oxalate and with that to 270 μg. of the platinichloride.

TABLE 5

Solution	Vol. injected c.c.	Wt. of crystals µg.	Arterial pressure, mm. Hg	
			Base-line	Rise
Oxalate from $NaHSO_3$(1)	0·3	54	134	27
Oxalate before $NaHSO_3$	0·4	52	129	30
Picrate	0·5	100	127	32
	0·4	80	126	29
Oxalate before $NaHSO_3$	0·5	65	125	33
Platinichloride	3·0	270	123	32

Solution A described in the text was inactive in a dose of 5 c.c.
Solution B was active in a dose of 2 c.c.

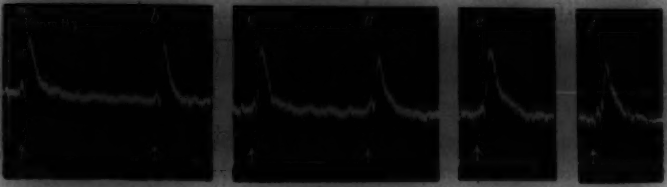


Fig. 8. The tracings show the responses to the following solutions: (a) 0·3 c.c. oxalate after first $NaHSO_3$ wash; (b) 0·4 c.c. oxalate before $NaHSO_3$ wash; (c) 0·5 c.c. picrate; (d) 0·4 c.c. picrate; (e) 0·5 c.c. oxalate before $NaHSO_3$ wash; (f) 3·0 c.c. platinichloride.

(c) *Analyses of the oxalate, picrate, and platinichloride*

The oxalate from the first bisulphite treatment, the picrate, and the platinichloride were sent for micro-analysis. The oxalate was dried in a high vacuum at room temperature, the picrate in a high vacuum at 100° C.

Oxalate: 3·562 mg. gave 5·890 mg. CO_2 and 1·610 mg. H_2O.
4·199 mg. gave 6·950 mg. CO_2 and 1·850 mg. H_2O.
3·22 mg. gave 0·171 c.c. N at 765/21°.
C 45%, 45·3%; H 5·0%, 4·9%; N 6·2%.

These results are in good agreement with those obtained for the oxalate of the similar pressor base obtained by the steam distillation process, and which showed the same melting-point. The empirical formula is again $C_8H_{11}NO_6$.

Picrate: 3·704 mg. gave 5·800 mg. CO_2 and 1·130 mg. H_2O.
3·700 mg. gave 5·820 mg. CO_2 and 1·080 mg. H_2O.
2·698 mg. gave 0·420 c.c. N at 765/21°.
C 42·6%, 43·0%; H 3·4%, 3·25%; N 18·2%.

Platinichloride: 2·319 mg. gave 1·810 mg. CO_2 and 0·550 mg. H_2O and 0·775 mg. residue.
2·677 mg. gave 0·127 c.c. N at 759/23°.
C 21·29%; H 2·637%; Pt 33·44%; N 5·48%.

The C and Pt percentages agree with those obtained for the platinichloride by the steam process, but the N value by the steam process was lower and the H higher. Simultaneous melting-point determinations on this platinichloride, the previous platinichloride, and the mixed salts showed a parallel decomposition between 275 and 280° C.

The results of analysis of a second preparation (30 October 1942) of the platinichloride from the oxalate were:

3·895 mg. gave 3·180 mg. CO_2 and 1·050 mg. H_2O and 1·258 mg. residue. C 22·3%; H 2·99%; Pt 32·29%.

The results of analysis of a third preparation (22 February 1943) of the platinichloride were:

4·003 mg. gave 0·169 c.c. N at 774/25°. N 4·91%.

These H and N figures are in close agreement with those for the platinichloride obtained from the second isolation by the steam distillation route.

(d) *The ultra-violet absorption spectrum of the base from the Pb filtrate*

It therefore remained only to study the ultra-violet absorption spectrum of the pressor base isolated from the Pb filtrate.

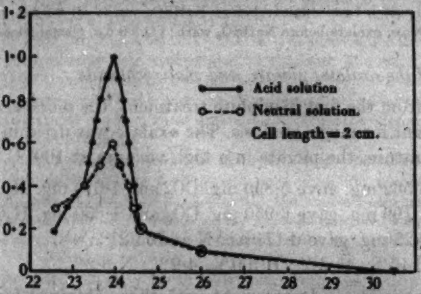


Fig. 9. The curves show the ultra-violet absorption of the base A obtained from decomposition of the oxalate formed from the filtrate of urine concentrate treated with basic lead acetate. Ordinates and abscissae as in Fig. 5.

0·7 mg. of the oxalate were dissolved in 10 c.c. 0·9% NaCl. This solution was tested for pressor activity on a cat, wt. 3·3 kg. 0·6 c.c. produced a rise in pressure of 35 mm. Hg from a base-line of 171 mm. Hg, and the response equated roughly with that to 10 μg. of adrenaline tartrate in 1·0 c.c. 0·9% NaCl. 4·0 c.c. of the oxalate solution were treated with 0·1 g. NaOH, brought to a volume of 5 c.c., and the solution was extracted three times with 10 c.c. ether, the combined ether extracts being shaken with 10 c.c. 2% HCl. The acid aqueous phase was boiled gently to dispel ether, cooled and brought to volume. The ultra-violet absorption spectrum was studied in acid solution, the solution was then neutralized to litmus by the addition of 40% NaOH, and the spectrum was again examined. Fig. 9 shows the results obtained. The base isolated from the Pb filtrate thus shows an absorption band at 2600 A., as does the base first isolated by the steam distillation route.

From the oxalate used for the ultra-violet absorption spectra, the platinichloride of 30 October 1942 was prepared. 3·0 mg. of this platinichloride were dissolved in 10 c.c. water and made alkaline with 1·0 c.c. 10% NaOH. This solution was thoroughly extracted three times with ether, and the combined ether extracts were shaken with 8 c.c. 2% HCl. After the ether had been dispelled by boiling, the solution was cooled, and brought to volume. 4·0 c.c. of the solution were neutralized to litmus by the addition of 0·2 c.c. 40% NaOH. To the remaining 4·0 c.c. of acid solution 0·2 c.c. water were added. After the absorption spectrum of the acid solution had been photographed, the solution was made alkaline by the addition of 0·1 g. NaOH and the spectrum again photographed. Control aqueous solutions were also prepared and similarly examined. The results are shown in Fig. 10.

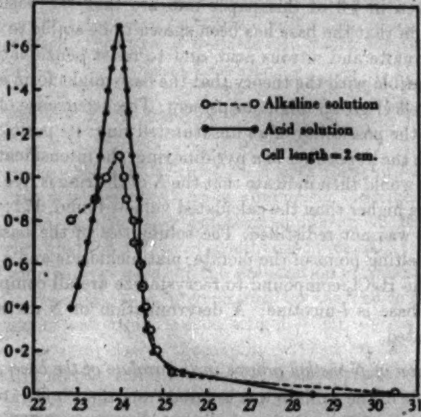


Fig. 10. The curves show the ultra-violet absorption of the base A obtained from decomposition of the platinichloride. Ordinates and abscissae as in Fig. 5.

The close agreement of the analyses of the oxalates and platinichlorides, the melting-point observations, and the ultra-violet absorption spectra, together show that the base A isolated from urine by the steam distillation process is identical with the similar base found in the filtrate of urine treated with basic lead acetate.

5. *A discussion of the platinichloride and picrate analyses in relationship to the findings in* § 3

The analytical results for the picrate and platinichloride, after drying at high vacuum, correspond closely with the percentages calculated for a diacidic base containing no O and having the formula $C_{10}H_{14}N_2$:

Picrate: Found—C 42·6%, 43·0%; H 3·4%, 3·25%; N 18·2%.
Calc.— C 42·58%; H 3·23%; N 18·06%.
Platinichloride: Found—C 21·3%, 22·3%; H 2·64%, 2·99%; Pt 33·4%, 32·29%; N 5·48%, 4·91%.
Calc.— C 20·98%; H 2·77%; Pt 34·07%; N 4·9%.

The results given in § 3 of this paper indicate that the basic N is all of a tertiary nature in that the base has been shown to be stable to treatment with sodium hypobromite and nitrous acid, and to resist benzoylation. There was evidence compatible with the theory that the base might form an amine oxide, and that an N-alkyl group might be present. The occurrence of an absorption band indicates the possibility of an unsaturated ring; its position at 2600 A. is compatible with the hypothesis of a pyridine ring; the intensification of the band in acid solution would then indicate that the N of the ring is free. The molecular weight finding is higher than the calculated value—found, 171; calculated, 162—but the base was not redistilled. The solubilities of the base, its volatility in steam, the melting-point of the picrate, platinichloride and aurichloride and the failure of the $HgCl_2$ compound to recrystallize are all compatible with the view that the base is *l*-nicotine. A determination of N-methyl groups was therefore indicated.

The determination of N-methyl groups in the oxalate of the base A

A fresh sample of the oxalate was isolated from the Pb filtrate, and was sent for the estimation of N-methyl and O-methyl groups. 6·767 mg. yielded no $—OCH_3$ groups, but gave 2·830 mg. AgI corresponding with 5·15% NCH_3. One of the two N atoms, therefore, carries a single methyl group.

6. *The isolation of* l-*nicotine from tobacco, as the picrate, and the preparation of the oxalate from the picrate*

For purposes of comparison, a small quantity of *l*-nicotine was isolated from tobacco, as the picrate, and purified by repeated recrystallization (m.p. 218° C.). From this picrate were prepared the oxalate, the *d*-tartrate, the *dl*-tartrate, and the platinichloride. Samples of the oxalate, picrate, and *d*-tartrate were sent for analysis:

Picrate: Found—C 42·58%; H 3·24%; N 18·1%.
Calc.— C 42·5%; H 3·23%; N 18·06%.
Oxalate: Found—C 43·5%, 43·5%; H 5·1%, 5·14%; N 5·99%.
Calc.— on $C_{10}H_{14}N_2.2(COOH)_2$. C 49·1%; H 5·26%; N 8·19% (see § 11).

This oxalate was prepared in the same manner as the oxalate of the base A, being precipitated from a dried ether solution by the addition of anhydrous oxalic acid in ether, recrystallized from absolute alcohol, and dried before analysis in a high vacuum at room temperature.

Optical rotation:

> 0·015767 g. of the oxalate in a water solution weighing 0·315731 g.—
> $\alpha_D^{21°} = +0{\cdot}21°$; $[\alpha]_D^{21°} = +13{\cdot}22°$, $l = 0{\cdot}5$ dm.

This oxalate is discussed in relationship to that of the base A under § 11.

The report of the analysis and optical rotation of the *d*-tartrate is given in subsection (*b*) of the next section.

7. *The isolation of the base A from the lead filtrate for comparison with* l-*nicotine*

(*a*) *The pressor activity of the anhydrous oxalate and picrate, and melting-point of the picrates*

In order to compare the salts of the base A with those of *l*-nicotine, 89 l. of normal human urine were worked up as previously described, as far as the final solution of the base in ether before precipitation of the oxalate. This ether solution was dried for 3 days over anhydrous Na_2SO_4. One-third of the decanted ether solution was used for the preparation of an anhydrous oxalate of the base A; the oxalate was precipitated by the cautious addition of a saturated solution of anhydrous oxalic acid in ether, excess being avoided. The precipitate was spun down, thoroughly washed on the centrifuge with ether, and dried in air for 1 hr.: 0·15 mg. of these crystals were dissolved in 10 c.c. 0·9% NaCl for examination of pressor activity, and the remainder was sent for micro-analysis, the results being given in § 11 of this paper. The remaining two-thirds of the dry ether solution of the base A were precipitated with a solution of picric acid in ether. The precipitate was spun down, and was washed four times on the centrifuge with ether. The yellow powdery residue was dried in air for 1 hr. 3·3 mg. were dissolved in 10 c.c. 0·9% NaCl, this solution being used in the test for pressor activity. One-half of the picrate remainder was recrystallized from hot water: the crystals were filtered off after 48 hr., washed with ice cold water, alcohol and ether, and dried in air for 1 hr. 0·6 mg. of the recrystallized picrate was dissolved in 10 c.c. 0·9% NaCl. Samples of each of these picrates were sent to Dr G. Weiler and were dried at high-vacuum and room temperature before analysis.

No. 6. Picrate not recrystallized:	C 42·6%, 43·1%; H 3·1%, 3·37%; N 18·6%, 18·1%.
No. 7. Recrystallized picrate:	C 44%; H 3·32%; N 17·3%.
Calc. for nicotine picrate:	C 42·58%; H 3·32%; N 18·06%.

These picrates were tested for pressor activity in a cat, wt. 3·0 kg., and the responses were equated with those to a solution of *l*-nicotine *dl*-acid tartrate. The pressor activity of this sample of *l*-nicotine tartrate in terms of its base content, was later compared several times with that of *l*-nicotine picrate from tobacco. 161 μg. *l*-nicotine picrate (tobacco) equated with 142 μg. of this acid tartrate, the theoretical correspondence being 161 μg. of picrate with 130 μg. of tartrate. The results obtained are shown in Fig. 11 and Table 6.

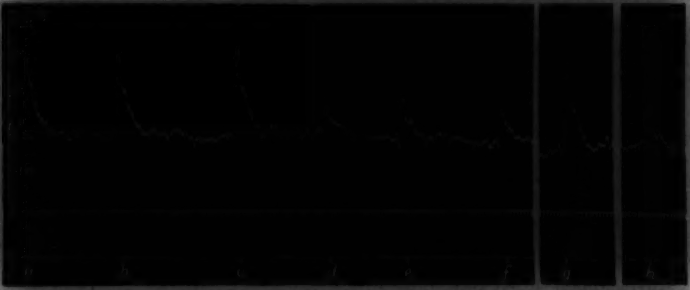


Fig. 11. The tracings show the responses to the following solutions: (*a*) 1 c.c. recrystallized picrate; (*b*) 0·2 c.c. anhydrous picrate; (*c*) 1·6 c.c. anhydrous oxalate; (*d*) 0·8 c.c. anhydrous oxalate; (*e*) 0·1 c.c. anhydrous picrate; (*f*) 0·5 c.c. recrystallized picrate; (*g*) 0·5 c.c. *l*-nicotine tartrate; (*h*) 0·25 c.c. *l*-nicotine tartrate. Time marker, 5 sec.

TABLE 6

Solution injected	Vol. injected c.c.	Wt. of crystals μg.	Arterial pressure, mm. Hg Base-line	Arterial pressure, mm. Hg Rise
Anhydrous picrate	0·2	66	173	46
	0·1	33	171	27
Recrystallized picrate	1·0	66	177	44
	0·5	30	179	19
Anhydrous picrate	0·1	33	175	17
Recrystallized picrate	1·0	60	161	42
Anhydrous picrate	0·2	66	155	44
Anhydrous oxalate	1·6	24	156	46
	0·8	12	157	18
Anhydrous picrate	0·1	33	155	22
Recrystallized picrate	0·5	30	152	15
l-Nicotine tartrate	0·5	71	149	45

The responses recorded in the lower part of this table are illustrated in Fig. 11.

The two picrates are of approximately equal activity. The melting-point of the anhydrous picrate after drying for 1 hr. in air was 225° C. 60–66 μg. of these picrates equate with 71 μg. of the acid tartrate of *l*-nicotine. Theoretically 66 μg. of *l*-nicotine picrate should equate with 54 μg. of *l*-nicotine acid tartrate-$2H_2O$. These picrates had, therefore, greater activity than would be expected for *l*-nicotine.

The picrates made from urine were then stored for 15 days in an evacuated desiccator over P_2O_5. At the end of this period a melting-point determination on the anhydrous picrate gave 217° C., and on the recrystallized picrate 218° C. These picrates were therefore again equated with *l*-nicotine. The *l*-nicotine used on this occasion was an anhydrous picrate prepared in the same manner as the picrate of the base A from the tartrate previously used, and the *l*-nicotine picrate isolated from tobacco. The melting-point determinations are given below:

l-Nicotine picrate (tobacco) 218° C.	Anhydrous picrate 217° C.
l-Nicotine picrate (tobacco) 218° C.	Recrystallized picrate 218° C.
l-Nicotine picrate (tobacco) 217° C.	*l*-Nicotine picrate from tartrate 217° C.
l-Nicotine picrate (tobacco) 218° C.	Mixed *l*-nicotine picrate (tobacco) and anhydrous picrate 217° C.
l-Nicotine picrate (tobacco) 217° C.	Mixed *l*-nicotine picrate (tobacco) and recrystallized picrate 216° C.

The following solutions were then prepared, and were tested for pressor activity in a cat weighing 1·6 kg.: (1) *l*-nicotine picrate (from tartrate) 1·03 mg., (2) *l*-nicotine picrate (tobacco) 2·75 mg., (3) anhydrous picrate (from urine) 1·0 mg., each dissolved in 10 c.c. 0·9% NaCl. The results are shown in Table 7.

TABLE 7

Solution	Vol. injected c.c.	Wt. of crystals μg.	Arterial pressure, mm. Hg	
			Base-line	Rise
2	0·2	54	118	10
	0·3	71	118	23
	0·4	108	118	26
3	0·75	75	114	16
	1·0	100	117	23
	0·8	80	117	18
2	0·3	71	118	19
3	0·8	80	118	19
2	0·3	71	113	15
	0·3	71	115	18
3	0·6	60	117	6
	0·7	70	115	10
	0·8	80	116	14
1	1·0	103	117	19
3	1·0	100	117	21

These picrates equated fairly nearly in pressor activity—71 μg. of tobacco picrate equates with 80 μg. of the anhydrous picrate; and 100 μg. of tobacco picrate is slightly more active than 103 μg. of the picrate made from the tartrate. The picrates from the base A were again stored in a vacuum to discover whether further change would occur. After 16 days there had been no alteration in the melting-points. The following solutions were then prepared and were tested on a cat, wt. 3·0 kg.: (1) *l*-nicotine picrate (tobacco) 2·3 mg., (2) anhydrous picrate (from urine) 1·4 mg., (3) *l*-nicotine picrate (from tartrate)

2·4 mg., each dissolved in 10 c.c. 0·9% NaCl. The results are summarized in Table 8 and illustrated in Fig. 12. After further treatment in a vacuum for 2 weeks the anhydrous picrate still equated with *l*-nicotine picrate, and there was no alteration in the melting-point.

Table 8

Solution	Vol. injected c.c.	Wt. of crystals µg.	Arterial pressure, mm. Hg Base-line	Arterial pressure, mm. Hg Rise
2	0·8	112	139	49
3	0·5	120	140	48
1	0·5	115	138	48
	0·25	57·5	136	22
3	0·25	60	132	28
1	0·25	57·5	130	30
2	0·4	56	130	28

Fig. 12. The tracings show the responses to the following solutions: (*a*) 0·8 c.c. soln. 2; (*b*) 0·5 c.c. soln. 3; (*c*) 0·5 c.c. soln. 1; (*d*) 0·25 c.c. soln. 1; (*e*) 0·25 c.c. soln. 3; (*f*) 0·25 c.c. soln. 1; (*g*) 0·4 c.c. soln. 2. Time marker, 5 sec.

(*b*) *The optical activity of the* d-*tartrates*

To confirm the identity of these vacuum-treated picrates with the picrate of *l*-nicotine, 133 l. of urine were worked up as previously described for the isolation of the base A from the Pb filtrate, as far as the final ether solution before precipitation of the oxalate. The ether solution was dried for 8 days over anhydrous Na_2SO_4 and one-half was then treated with a few drops of a saturated solution of *d*-tartaric acid in ethyl alcohol. The precipitate forming was spun down, and recrystallized from 70% alcohol. The crystals forming were washed with ice-cold alcohol, then with ether, and dried in a vacuum at room temperature, over soda lime.

The *d*-tartrate of *l*-nicotine was similarly prepared, by extracting the *l*-nicotine from an alkaline solution of the oxalate with ether, drying the ether, and precipitating, recrystallizing, and drying as described above.

Samples of these two salts were sent for micro-analysis and determination of optical activity:

Acid tartrate of the base A. No. 14, 10 June 1943

4·179 mg. gave 6·740 mg. CO_2 and 2·240 mg. H_2O; C 44%, H 5·9%.
Calc. on $C_{10}H_{14}N_2 . 2C_4H_6O_6 . 2H_2O$; C 43·8%, H 6·0%.

Optical rotation: 0·010692 g. in 0·311566 g. solution (water):

$$\alpha_D^{20^\circ} = +0{\cdot}36^\circ,\ [\alpha]_D^{20^\circ} = +21{\cdot}2^\circ,\ l = 0{\cdot}5\ \text{dm.}$$

Acid tartrate of nicotine. No. 17, 22 September 1943

$$\left.\begin{array}{l}\text{4·485 mg. gave 7·160 mg. } CO_2 \text{ and 2·420 mg. } H_2O\\ \text{4·334 mg. gave 6·910 mg. } CO_2 \text{ and 2·380 mg. } H_2O\end{array}\right\}\begin{array}{l}\text{C 43·5\%, 43·45\%;}\\ \text{H 5·85\%, 6·1\%.}\end{array}$$

Optical rotation: 0·020108 g. in 0·421808 g. solution (water):

$$\alpha_D^{18^\circ} = +0{\cdot}54^\circ,\ [\alpha]_D^{18^\circ} = +21{\cdot}6^\circ,\ l = 0{\cdot}5\ \text{dm.}$$

These tartrates were now tested for pressor activity on a cat, wt. 1·3 kg. 160 μg. of the tartrate of the base A gave a rise in blood pressure of 57 mm. Hg from a base-line of 70 mm. Hg. 165 μg. of *l*-nicotine tartrate gave a rise of 64 mm. Hg from a base-line of 69 mm. Hg.

(c) *The melting-points of the platinichlorides of* l-*nicotine and of the base A from urine*

The preparation of the platinichloride used for these determinations is described in § 4 of this paper. The sample used was analysed: found N 4·91%; calc. for *l*-nicotine 4·9%. A comparison was made of the melting-points of *l*-nicotine platinichloride, the platinichloride of the base A, and a mixture of these two compounds, the observations being made simultaneously, since the melting-points were not sharp, were accompanied by decomposition, and varied with the rate of heating. The three decomposed similarly between 279 and 282° C.

The base isolated from urine, as the oxalate and the picrate, had a higher pressor activity than *l*-nicotine. By simply submitting the picrate to drying in a vacuum over P_2O_5, the activity fell to equal that of *l*-nicotine and was not further altered by prolonged drying. The analyses of the picrate after drying in a high vacuum are in good agreement with those calculated for *l*-nicotine picrate, and the mixed melting-points of the picrates after vacuum treatment confirm the identity of the vacuum-treated picrate of the base A with *l*-nicotine. The optical activity of the *d*-tartrate of the base A corresponds with that of the *d*-tartrate of *l*-nicotine, and the mixed melting-points of the platinichlorides again confirm the identity of the less active form of the base A with *l*-nicotine.

The experiments reported so far, whilst clearly demonstrating the relationship of a pressor base found in human urine, to *l*-nicotine, do not show whether this base may be of physiological significance, because the urine was collected from male convalescent patients at a hospital annex where smoking was allowed within restricted hours. A search for this base was therefore made in the urine of female surgical patients, who were confined to bed in wards where smoking was not permitted, and also in the urine of dogs.

8. *The presence of the base A in the urine of non-smokers*

(*a*) *Preliminary experiment indicating the presence of the base A*

27 l. of the women's urine were worked by the Pb precipitation method, and the procedure followed was that previously described for the isolation of the oxalate of the base A from the filtrate. On the addition of a solution anhydrous oxalic acid in ether to the dried ether which would contain the base A if present, the typical generalized white turbidity was seen. The precipitate was spun down, washed with ether, dried, and dissolved in 30 c.c. 0·9% NaCl.

This solution was tested for pressor activity on a cat, wt. 2·3 kg., and prepared as before described. The pressor response obtained was equated with that to a solution of *l*-nicotine acid tartrate in 0·9% NaCl, containing 120 μg./c.c. The results are summarized in Table 9.

TABLE 9

Solution	Vol. injected c.c.	Arterial pressure, mm. Hg	
		Base-line	Rise
Urine oxalate	1·5	162	44
l-Nicotine acid tartrate	1·5	159	42
Urine oxalate	1·0	164	33
l-Nicotine acid tartrate	1·0	158	35

(*b*) *The isolation of the base A as oxalate and picrate*

126 l. of urine from the same source were collected in the course of 6 days. This urine contained a considerable amount of blood. It was worked up by the method previously described for the isolation of the oxalate of the base A from the filtrate of urine concentrate treated with basic lead acetate. A low yield of oxalate, 7 mg., was obtained. This oxalate after recrystallization from alcohol and drying in air, had m.p. 96° C. After drying in a high vacuum at room temperature, the m.p. was 107° C.

1·0 mg. of the oxalate was dissolved in 20 c.c. 0·9% NaCl and was tested for pressor activity (see Table 10). The remainder was converted to the picrate, which was recrystallized from hot water. These crystals after drying in a high vacuum at 40° C. had m.p. 217° C.: that of *l*-nicotine picrate was 218° C. and that of a mixture, 217° C.

(*c*) *A control experiment to confirm the finding of small quantities of the base A in the urine of non-smokers*

The yield of oxalate obtained in the previous experiment was lower than that to be expected from a similar volume of the urine of the male patients who were permitted to smoke. 10 l. of the women's urine containing no blood were again worked by the Pb filtrate process as far as the final ether solution. This ether solution was shaken three times with 10 c.c. 0·66% HCl, the combined aqueous-acid extracts were gently boiled, and were neutralized with NaOH to a volume of 30·1 c.c. forming solution A.

The solution of the oxalate obtained under § 8 (*b*) and solution A were tested for pressor activity on a cat, wt. 3·0 kg. The results are recorded in Table 10.

TABLE 10

Solution	Vol. injected c.c.	Wt. of crystals µg.	Arterial pressure, mm. Hg	
			Base-line	Rise
A	3·0	—	160	43
Oxalate	0·9	45	137	23
	1·2	60	141	37

The ultra-violet absorption spectrum of solution A showed the presence of a band at 2600 A.

From these results it will be seen that approximately 1 cat pressor dose of the base A, i.e. the amount giving a rise in pressure of the order of 40 mm. Hg, was obtained from each litre of this urine. The yield, although a little higher than that of the two preceding experiments, is still lower than that obtained from the men's urine by the same route, which yielded 3 cat pressor doses for each litre of urine. From the men's urine by the steam route 3–4 cat pressor doses were obtained from each litre, a dose having roughly the same activity as 40 µg. *l*-nicotine oxalate. In order finally to identify this pressor base from the urine of non-smokers with *l*-nicotine, the picrate was isolated and analysed, melting-point determinations were made, the pressor response to the picrate was equated in a cat with that to *l*-nicotine, and the ultra-violet absorption of the two bases were compared.

(*d*) *The isolation from the urine of non-smokers of the base A, and its identification with* l-*nicotine*

(α) *Isolation as the picrate, and analysis.* In the course of 2 weeks 139 l. of women's urine from the same source were collected. The method of working up this urine followed closely that described for the isolation of the oxalate of the base A from the filtrate of urine concentrate treated with basic lead acetate, described above, as far as the final ether solution before the precipitation of the oxalate. The ether solution was dried for 3 days over anhydrous Na_2SO_4. The ether was decanted, and treated with a solution of picric acid in ether. The precipitate which formed was spun down, and washed twice on the centrifuge with ether. 5 mg. of this picrate were reserved as picrate A: its m.p. was 210° C., and it left a small ash. The rest of the picrate was recrystallized from alcohol-ether mixture: the yield of this picrate, B, was approximately 9 mg. Both picrates were dried in a high vacuum at room temperature for 6 hr. The melting-point determinations on picrate B gave *l*-nicotine picrate

217–218° C., picrate B 217° C., mixed m.p. 216° C. Samples of both picrates were sent for analysis.

A. 3·151 mg. gave 3·740 mg. CO_2, 0·760 mg. H_2O, 0·627 mg. residue,

B. 1·148 mg. gave 1·830 mg. CO_2, 0·320 mg. H_2O, no residue,
3·791 mg. gave 0·581 c.c. N at 767/21°.

Thus we have for: Picrate B, found, C 43·58%; H 3·1%; N 17·9%; *l*-nicotine picrate, calc., C 42·58%; H 3·23%; N 18·06%.

(β) *The picrate of the base A, equated with* l-*nicotine picrate.* The following solutions were prepared. 0·48 mg. picrate A, 0·5 mg. picrate B, and 1·65 mg. *l*-nicotine picrate, each dissolved in 10 c.c. 0·9% NaCl, and an unknown weight of picrate A dissolved in 20 c.c. 0·9% NaCl. The pressor activities of these solutions were compared in a cat, wt. 2·65 kg., and the results are summarized in Table 11. Picrate B had the same activity as *l*-nicotine picrate. 3·0 c.c. of the second picrate A solution had approximately the same activity as 300 μg. of *l*-nicotine picrate.

TABLE 11

Solution	Vol. injected c.c.	Wt. of crystals μg.	Arterial pressure, mm. Hg	
			Base-line	Rise
Picrate A	3·3	158	133	30
l-Nicotine picrate	1·0	165	130	42
Picrate A	3·3	158	127	35
Picrate B	3·3	165	125	45
l-Nicotine picrate	1·0 in 3·3	165	127	45
Cat decerebrated				
Picrate A, 2nd solution	3·0	—	54	117
l-Nicotine picrate	6·0 of 1 in 3·3 dilution	300	59	112

(γ) *Comparison of the ultra-violet absorption spectra of* l-*nicotine and the base A.* In order to compare the ultra-violet absorption spectrum of this base from urine with that of *l*-nicotine, the following procedure was adopted.

9·0 c.c. of the second solution of picrate A were treated with 0·1 g. NaOH, and were thoroughly extracted three times with ether. The ether extract was washed with an aqueous solution of 1% NaOH, the ether being then shaken with 6·0 c.c. 1% HCl.

9·0 c.c. of a 6·0 in 9·9 c.c. dilution of a solution of *l*-nicotine picrate, 1·65 mg. in 10 c.c., were similarly treated.

These solutions were freed of ether by warming in a water-bath at 100–90° C. for 15 min. and were then brought to a volume of 6·0 c.c. 3·0 c.c. of each solution were cautiously treated with powdered NaOH until just alkaline to litmus. Control solutions were similarly prepared, from 1% HCl. The ultra-violet absorption spectra of *l*-nicotine and the base from the urine of non-smokers were compared in alkaline and acid solution: the results are shown in Fig. 13, and are in very close agreement. The cell length was 1·0 cm.

There is therefore no doubt that the same base A, isolated as *l*-nicotine, is present in the urine of patients who have had no opportunity to smoke.

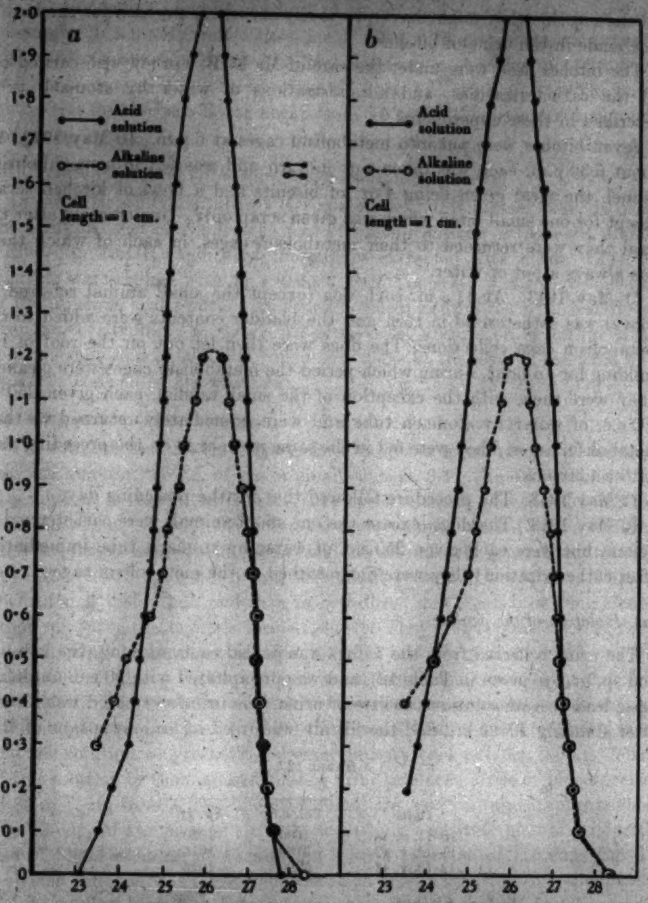


Fig. 13. (*a*) The ultra-violet absorption curves given by *l*-nicotine. (*b*) The corresponding curves given by the base A obtained from non-smokers' urine. Ordinates and abscissae as in Fig. 5.

9. *The presence of the base A in dog urine*

In order to confirm the presence of the base A in the urine of non-smokers, and lest some of the patients should have smoked heavily before admission to the wards, and not have excreted in their few pre-operative days in hospital all the nicotine they might have previously absorbed, a search for the base A was made in the urine of bitches.

The bitches used were under the care of Mr E. B. Verney who carried out all the catheterizations, and administrations of water by stomach tube, described in these experiments.

Seven bitches were put into metabolism cages at 6 p.m., 10 May 1943. At about 6·30 p.m. each was taken out in turn and was fed in a neighbouring kennel, the meal given being 4 oz. of biscuits and a bowl of kitchen scrap, except for one small bitch which was given scrap only. Immediately after the meal they were returned to their metabolism cages, in each of which there was always a pot of water.

11 May 1943. At 11 a.m. each dog (except the small animal referred to above) was catheterized in turn and the bladder contents were added to the metabolism cage collections. The dogs were then let out on the roof of the building for an hour, during which period the metabolism cages were cleaned. They were then, with the exception of the small animal, each given 250 or 300 c.c. of water by stomach tube and were immediately returned to their metabolism cages. They were fed in the same manner as on the preceding day, but at 4.30 p.m.

12 May 1943. The procedure followed that for the preceding day.

13 May 1943. The dogs (except the one small animal) were catheterized as before, but were each given 250 c.c. of water by stomach tube immediately after catheterization: they were not returned to the metabolism cages.

(a) *Isolation of the oxalate*

The urine collected from the 7 dogs was pooled each morning (the volumes and sp. gr. are given in Table 12), and was precipitated with 50 c.c. unfiltered basic lead acetate solution per litre of urine. The urine so treated was filtered after standing 20–26 hr., and the filtrate was freed of lead by means of H_2S.

TABLE 12

	Urine	
Date	Vol. c.c.	Sp. gr.
10–11. v. 43	1600	1030
11–12. v. 43	3150	1015
12–13. v. 43	3200	1015

The combined Pb-free filtrates were concentrated to a final volume of 1·3 l. and, after cooling, the concentrate was filtered by suction through kieselguhr. The concentrate was then made alkaline, and worked up according to the scheme given in § 4 of this paper, as far as the first precipitation of the oxalate before recrystallization. On the addition of the solution of anhydrous oxalic acid in ether, the typical white turbidity appeared. The precipitate was spun down, washed with ether, and was dissolved in 25 c.c. 0·9% NaCl. 1·5 c.c. of this solution when injected into a cat, wt. 3·0 kg., produced a rise of blood pressure of 34 mm. Hg from a base-line of 149 mm. Hg, thus equating roughly with a rise in blood pressure of 36 mm. Hg from a base-line of 146 mm. Hg which was produced by the injection of 70 μg. *l*-nicotine picrate. The colorimetric test for the base B was negative on 0·5 c.c. of this base A solution from dog urine.

(*b*) *Preliminary study of the ultra-violet absorption spectrum*

In order to study the ultra-violet spectrum of this pressor base, 10 c.c. of the solution used above for the test of pressor activity were made alkaline with 0·1 g. NaOH, and were thoroughly extracted with ether. The ether extracts were shaken with 5 c.c. 0·66% HCl. The ether was gently boiled out of the aqueous acid solution which was cooled, neutralized with NaOH, and brought to a volume of 6 c.c. An ultra-violet absorption spectrum of this solution showed a band at approximately 2600 A., the peak of the band being at *d* 0·6. The cell length was 1 cm.

(*c*) *Preparation of the picrate*

The solution used for the ultra-violet absorption spectrum was added to the residual amount, 9·8 c.c. of the original solution, 0·1 g. NaOH was added and the solution extracted with ether. The ether extracts were dried for 8 days over anhydrous Na_2SO_4. The ether was decanted and treated with a few drops of a saturated solution of picric acid in alcohol. A generalized turbidity appeared, which spun down to a very small volume of precipitate. This was washed well with ether, and then recrystallized in a small tube from alcohol-ether mixture: the crystals formed slowly. They were spun down as far as possible, and the supernatant decanted. The crystals were washed twice with ether, and dried in a high vacuum at room temperature over P_2O_5: their m.p. was 217° C. The available amount of the crystals was too small for accurate weighing, but approximately 140–145 μg. were dissolved in 7 c.c. 0·9% NaCl, and the solution was tested for pressor activity on a cat, wt. 2·5 kg. 6·0 c.c. of this solution of the picrate from dog urine produced a rise of blood pressure of 33 mm. Hg from a base-line of 129 mm. Hg, and contained approximately 120–125 μg. of the picrate. In this cat 165 μg. *l*-nicotine picrate produced a rise of blood pressure of 44 mm. Hg from a base-line of 128 mm. Hg. The response obtained was therefore of the same order as that to *l*-nicotine picrate.

10. *A repeat isolation of the base A from dog urine for comparison with* l-*nicotine*

In order to compare the pressor activity of this base from dog urine with that of *l*-nicotine the following experiment was carried out. The dogs were again under the care of Mr E. B. Verney, who carried out the catheterizations and administrations of water by stomach tube. Six bitches were used, and the procedure was essentially the same as for the previous collection of dog urine, but catheterization and administration of water were restricted to those animals which had not emptied their bladders during the 2½ hr. preceding their hour on the roof. The urine collection began at 3 p.m. on 30 August 1943 and ended at 12 noon on 4 September 1943, and the daily volumes etc. are given in Table 13.

TABLE 13

Date	Urine		Vol. basic lead acetate soln. added, c.c.
	Vol. c.c.	Sp. gr.	
30-31. viii. 43	2580	1014	160
– 1. ix. 43	2690	1012	160
– 2. ix. 43	1415	1032	120
– 3. ix. 43	1640	1028	160
– 4. ix. 43	2080	1022	160

(a) *Isolation of the oxalate*

The urine collected during the preceding 24 hr. was pooled, and was precipitated with a basic lead acetate solution similar to that described in the last experiment. After standing 24 hr. the precipitate was filtered off, and the filtrate was freed of Pb by means of H_2S. The Pb-free filtrates were then concentrated to a final volume of 2·1 l. This Pb-free concentrate was then worked up according to the method given in § 4 of this paper, as far as the first ether extraction; the base was passed back from ether solution into 20 c.c. 0·64 % HCl, the ether was gently boiled out of the aqueous phase, which was cooled and neutralized with NaOH to a volume of 23 c.c.

(α) *A preliminary comparison of pressor activity and ultra-violet absorption of the base A and* l-*nicotine.* The solution was tested for pressor activity on a cat, wt. 2·1 kg., and the activity was compared with that of a control solution of *l*-nicotine hydrochloride. This was prepared by ether extraction of 45 c.c. of an alkaline solution of *l*-nicotine *d*-acid tartrate-$2H_2O$ containing approximately 12 mg./100 c.c. The ether extracts were shaken with 6 c.c. 0·64 % HCl, the ether being gently boiled out of the acid solution which was neutralized with NaOH to a volume of 6·3 c.c. The results obtained are shown in Table 14.

TABLE 14

Solution	Vol. injected c.c.	Arterial pressure, mm. Hg	
		Base-line	Rise
From dog urine	0·1	134	7
	0·3	134	14
	0·5	133	29
Nicotine control	0·2	129	26

From these results it may be seen that 0·2 c.c. of the nicotine control solution had a pressor activity of the same order as had 0·5 c.c. of the solution made from dog urine. For comparison of the ultra-violet absorption spectra of these solutions, 0·5 c.c. of the dog urine solution was diluted to 3·3 c.c. with 0·9 %

NaCl, and 0·4 c.c. of the nicotine control solution was diluted to 6·6 c.c. with 0·9% NaCl. The ultra-violet absorption spectra of these dilutions were photographed using a 2 cm. cell, and 0·9% NaCl as the control solution. The dilution from dog urine showed a band at approximately 2600 A., the peak of the band being at *d* 0·5. The nicotine control solution showed a similar band, the peak being at *d* 0·4.

The remaining solution from dog urine was made alkaline by the addition of 2 c.c. 10% NaOH, and was extracted thoroughly with ether. The ether extracts, volume approximately 40 c.c., were dried over anhydrous Na_2SO_4 for 3 days; the ether solution was decanted, and was treated with a solution of anhydrous oxalic acid in ether. The precipitate was spun down, was washed on the centrifuge with ether, and was dissolved in 10 c.c. of distilled water.

(*b*) *The preparation of solutions of the hydrochloride of the base A and of* l-*nicotine, showing similar absorption in the ultra-violet, for determination of their pressor activity*

To 2·0 c.c. of the oxalate solution, whose formation has just been described, were added 4·5 c.c. water and 0·5 c.c. 10% NaOH. This solution was thoroughly extracted with ether, and the ether extracts were shortly washed with 0·1% NaOH, and were then shaken with 6 c.c. 1% HCl. The acid aqueous solution was freed of ether by warming in a water-bath, and brought to a volume of 6·3 c.c. 3 c.c. of this were neutralized with NaOH and brought to a volume of 6 c.c.; and 3 c.c. were diluted with 1% HCl to a volume of 6 c.c.

A nicotine hydrochloride solution was prepared similarly from *l*-nicotine *d*-tartrate and contained 15·7 mg. of the acid salt in 20 c.c. The final solutions, each 10 c.c. in volume, corresponded to 7·85 mg. in 1% HCl and 7·85 mg. in 0·85% NaCl. The ultra-violet absorption spectrum of a 1/10 dilution of the neutral solution compared very closely with that of the neutral solution from dog urine. The acid solutions also gave strikingly similar spectra. The results are shown in Fig. 14, the control solutions being 0·85% NaCl and 1% HCl.

The two neutral solutions, having identical ultra-violet absorption spectra, were then compared as to pressor activity in a cat, wt. 1·3 kg. The results are illustrated in Fig. 15. The two solutions equated well.

The experiments described in §§ 9 and 10 of this paper show that the base A, isolated as *l*-nicotine, occurs also in the urine of bitches, and in an amount comparable with that found in the urine from male patients who were permitted to smoke. About 3 cat pressor doses per litre of urine from either source were obtained.

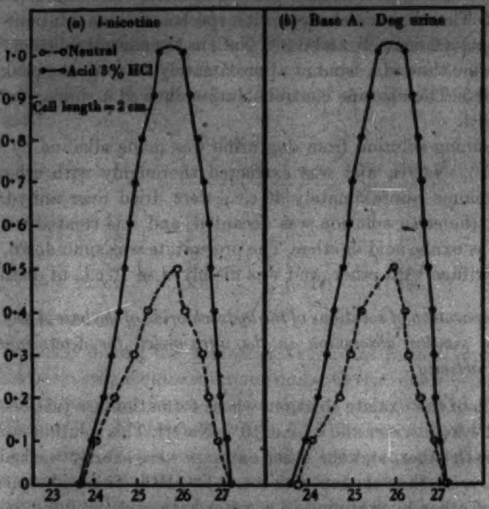


Fig. 14. (*a*) The ultra-violet absorption curves given by *l*-nicotine in acid and neutral solution. (*b*) The corresponding curves given by the base A obtained from dog urine. Ordinates and abscissae as in Fig. 5.

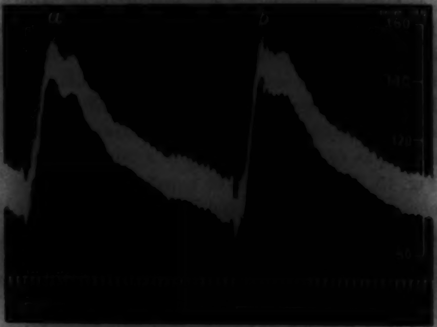


Fig. 15. The tracings show the responses to the following solutions: (*a*) 3 c.c. of the *l*-nicotine solution, (*b*) 3 c.c. of the solution of the base A from dog urine, the ultra-violet absorption spectra of which are illustrated in Fig. 14 *a*, *b* respectively.

11. *A discussion of the oxalate of the base A*

The oxalate analyses of the base A from urine correspond to a formula of $C_{16-17}H_{20-22}N_2O_{11-12}$. The percentages found are:

C 45·26, 44·4, 45·6, 44·9, 44·8, 45, 45·3;
H 5·64, 5·54, 5·4, 5·1, 5·3, 5·0, 4·9;
N 6·9, 6·8, 6·3, 6·1, 6·4, 6·2.

In order to exclude the possible presence of either water or alcohol of crystallization the anhydrous oxalate was isolated from the filtrate as described in § 7. The final ether solution of the base was dried for 4 days over three changes of anhydrous Na_2SO_4, and was then precipitated by the cautious addition of a saturated solution of anhydrous oxalic acid in ether. The precipitate was spun down, the supernatant ether was decanted, the precipitate was washed three times on the centrifuge with ether, and dried in air. This precipitate was sent for analysis:

3·951 mg. gave 6·715 mg. CO_2 and 2·120 mg. H_2O.
2·850 mg. gave 0·158 c.c. N at 764/24°.
C 46·34%; H 5·97%; N 6·38%.

This analysis corresponds with a formula of $C_{17}H_{24}N_2O_{11}$. The high C, H and O values cannot therefore be associated with the presence of alcohol or water of crystallization. 1·5 mg. of this oxalate were dissolved in 10 c.c. 0·9% NaCl, and were compared in pressor activity with a solution of *l*-nicotine *dl*-acid tartrate, and with the picrate, anhydrous and recrystallized, obtained at the same time (see p. 214). The results are recorded in Table 6. 24 μg. of this oxalate roughly equated with 66 μg. of the picrate and 71 μg. of the *l*-nicotine acid tartrate $2H_2O$. This oxalate was therefore remarkably active. Were the pressor activity due to the *l*-nicotine content of these crystals, 71 μg. of *l*-nicotine acid tartrate $2H_2O$ would be expected to equate with 87 μg. of *l*-nicotine picrate and 63 μg. of *l*-nicotine oxalate prepared by this method.

Some of the recrystallized oxalate of the base A dried in a high vacuum at room temperature and prepared for the determination of N-methyl groups (§ 6) was used to estimate the oxalic content of this oxalate, by precipitation of the oxalic acid as the Ca salt, washing once with water, and titrating in acid solution with 0·01 *N* $KMnO_4$. The procedure followed that described by Kolthoff & Furman (1929):

7 mg. of the oxalate were found to contain 3·712 mg. oxalic acid = 53% oxalic acid.

2·2 mg. of the oxalate were found to contain 1·158 mg. oxalic acid = 52·6% oxalic acid.

0·5 c.c. of a solution of cryst. oxalic acid—$2H_2O$, 0·7 g./50 c.c., was found to contain 5·07 mg. oxalic acid.

These results correspond to a formula of $C_{11-12}H_{15-17}N_2O_{1-2}(COOH)_5$.

The oxalate of *l*-nicotine was therefore prepared by the same method of precipitation, recrystallized from alcohol and dried in a high vacuum at room temperature (§ 6):

8·2 mg. were found to contain 5·2 mg. oxalic acid = 63·5 %.

4·2 mg. were found to contain 2·5 mg. oxalic acid = 60 %.

0·5 c.c. of a solution of oxalic acid, similar to that previously described, was found to contain 4·96 mg. oxalic acid.

The calculated oxalic acid content based on a formula of $C_{10}H_{14}N_2.3(COOH)_2$ for the oxalate of *l*-nicotine prepared by this method is 63 %.

A sample of this nicotine oxalate was sent for analysis.

Found: 3·616 mg. gave 5·770 mg. CO_2 and 1·660 mg. H_2O.
3·910 mg. gave 6·270 mg. CO_2 and 1·800 mg. H_2O.
3·358 mg. gave 0·176 c.c. N at 760/26°.
C 43·5 %, 43·5 %; H 5·1 %, 5·14 %; N 5·99 %.

The percentages calculated on $C_{10}H_{14}N_2.3(COOH)_2$ are: C 44·4, H 4·6, N 6·5. The figures are in poor agreement. A rough assay was therefore made of the *l*-nicotine content of this oxalate. The oxalate was equated in solution with *l*-nicotine picrate and *l*-nicotine *d*-acid tartrate $2H_2O$, in a cat, wt. 1·3 kg. The solutions were prepared as follows: (A) 1·0 mg. *l*-nicotine picrate, (B) 2·2 mg. *l*-nicotine *d*-acid tartrate $2H_2O$, (C) 5·7 mg. *l*-nicotine oxalate (as described above), each being dissolved in 10 c.c. 0·9 % NaCl. The results are given in Table 15.

TABLE 15

Solution	Vol. injected c.c.	Wt. of crystals µg.	Arterial pressure, mm. Hg	
			Base-line	Rise
B	0·7	154	75	53
	0·54	119	66	39
	0·65	143	67	52
	0·75	165	64	65
	0·75	165	62	64
A	2·0	200	60	68
B	0·75	165	61	70
B	0·75	165	63	52
C	0·3	171	58	66
B	0·85	187	59	52
C	0·275	156	55	51
C	0·3	171	58	63
C	0·275	156	62	52

200 µg. of *l*-nicotine picrate are theoretically equivalent to 162 µg. of the tartrate. 165 µg. of the tartrate were found to be approximately equivalent to 200 µg. of the picrate.

156 µg. of the oxalate were found to be approximately equivalent to 187 µg. of the tartrate, the *l*-nicotine content of the latter being 51 µg. If 51 µg. of *l*-nicotine be assumed present in 156 µg. of the oxalate, the oxalic acid content of the molecule is approximately 66 %, confirming the chemical finding of 63 %.

This *l*-nicotine oxalate was therefore recrystallized from dilute alcohol, and was dried in a high vacuum at room temperature. Re-estimation of the oxalic acid content now gave 46·3%. Some of this recrystallized oxalate was sent for analysis. The compound was again dried in a high vacuum at room temperature before the determinations were made:

3·973 mg. gave 6·475 mg. CO_2 and 1·880 mg. H_2O.
4·373 mg. gave 7·165 mg. CO_2 and 1·940 mg. H_2O.
2·960 mg. gave 0·195 c.c. N at 757/23°.
2·927 mg. gave 0·187 c.c. N at 761/21°.
C 43·4%, 44·6%; H 5·6%, 4·9%; N 7·56%, 7·45%.

The percentages calculated on $C_{10}H_{14}N_2.2(COOH)_2.2H_2O$ are C 44·5, H 5·84, N 7·4. It therefore seems probable that under the conditions of the precipitation and recrystallization from absolute alcohol, used throughout for the preparation of the oxalate of the base A, an atypical oxalate of *l*-nicotine is obtained, and that from this atypical oxalate the typical oxalate $C_{10}H_{14}N_2.2(COOH)_2.2H_2O$ is obtained by recrystallization from aqueous alcohol. It is likely that the oxalate of the base A is also atypical, or that it may be a mixture of the typical and the atypical type of oxalate.

12. *Discussion of the discrepancies arising if the original form of the base A is to be considered as* l-*nicotine*

Although these results may in part be the explanation of the analytical findings on the oxalate of the base A, they do not explain the very high pressor activity of some samples of this oxalate, notably that of the anhydrous oxalate described above. In another experiment already recorded (p. 209) 54 μg. of the recrystallized oxalate were found to equate with 80 μg. of the picrate which had been prepared from the oxalate, and recrystallized from dilute alcohol. The melting-point of the picrate was 218° C. [nicotine picrate 218° C.]. The analyses of this oxalate corresponded tolerably well with the formula $C_{10}H_{14}N_2.3(COOH)_2$:

Found: C 45·0%, 45·3%; H 5·0%, 4·9%; N 6·2%.
Calc.: C 44·4% ; H 4·65% ; N 6·5%.

80 μg. of *l*-nicotine picrate should theoretically equate with 56 μg. of the oxalate $C_{10}H_{14}N_2.3(COOH)_2$, this agreeing well with the experimental finding, viz. 54 μg. of the oxalate.

Moreover, abnormally high activity has also been found in an anhydrous and in a recrystallized picrate (see p. 214); and by the simple procedure of drying these two picrates in a high vacuum at room temperature the activity fell to equate accurately with that of *l*-nicotine picrate, and the analytical figures after such drying corresponded very closely with the calculated results for *l*-nicotine picrate. If this increased activity be due to a second pressor compound, it can be present only in minute traces, and having, therefore, extreme activity; it would not then be expected to recrystallize in a constant proportion with *l*-nicotine picrate; it would also have to be a highly volatile compound. It therefore seems possible that the original form of the base A is not *l*-nicotine, but is closely related to *l*-nicotine, is more active than *l*-nicotine, and is unstable.

No further useful attempt can be made to discuss this more active form of the base A until other investigations have been completed, but experiments in which *l*-nicotine *dl*-acid tartrate was added to urine concentrate are of interest at this stage. When moderate amounts of *l*-nicotine tartrate were added to urine concentrate, and the treated concentrate was worked up in parallel with the untreated concentrate, all the *l*-nicotine was apparently precipitated by the basic lead acetate. Only when large amounts of *l*-nicotine tartrate were added, was a small proportion found to be present in the final solution. These experiments are therefore briefly reported below. They again suggest the possibility that the actual form in which the base A is excreted differs from *l*-nicotine.

13. *The precipitation of* l-*nicotine by basic lead acetate, and its adsorption by lead sulphide*

(1) Urine concentrate, vol. 1500 c.c., corresponding to 18 l. of urine from male convalescent patients, was divided into six fractions of 250 c.c. To each of three of these fractions 5 mg. *l*-nicotine tartrate were added in 10 c.c. water. Each fraction was treated with 75 c.c. basic lead acetate solution, and after having been stirred was left to stand 48 hr. Those treated with nicotine were labelled N, and the control concentrates were labelled M. M_1 and N_1, M_2 and N_2, M_3 and N_3 were worked up separately but simultaneously. The concentrates were worked up exactly in the manner described for the isolation of the base A from the lead filtrate, given in § 4, as far as the first ether extraction. These ether solutions were shaken with 10 c.c. 0·64% HCl, the ether was removed from the acid aqueous phase by gentle boiling, and after cooling, 0·1 g. NaOH was added. The solutions were neutralized with HCl, and were brought to volume. These solutions were tested for pressor activity on a cat, wt. 3·0 kg. The results are recorded in Table 16, and show that the pressor activity of the final solutions from the *l*-nicotine-treated concentrate were not greater than those of the untreated concentrate.

(2) In order to demonstrate the absence of factors limiting the amount of *l*-nicotine which could reach the terminal solutions, this experiment was repeated, using 10 mg. of *l*-nicotine tartrate. Control final extractions and elutions were performed at each step. The results obtained were similar to those of the preceding experiment, and the control solutions shewed no loss of pressor activity at any stage.

TABLE 16

Solution	Vol. injected c.c.	Arterial pressure, mm. Hg	
		Base-line	Rise
M_1	0·1	173	11
N_1	0·1	172	15
M_2	0·1	169	20
N_2	0·1	168	19
M_3	0·1	167	22
N_3	0·1	164	22

(3) When, however, much larger quantities of *l*-nicotine tartrate were added, a little of the nicotine reached the final solution. When 54 mg. *l*-nicotine tartrate were added to concentrate corresponding to 6·6 l. of urine, untreated concentrate from a further 6·6 l. of urine being worked up in parallel, with full controls to exclude losses at each stage, an increase of pressor activity was found in the terminal solution from the *l*-nicotine-treated concentrate as compared with the untreated concentrate. The increase in activity corresponded to one-seventh of the activity of the added nicotine tartrate. The control solutions showed a very small loss of pressor activity at one stage only, and this only in the case of the *l*-nicotine-treated concentrate. Decomposition of the basic lead acetate precipitates from the treated and untreated concentrates resulted in a strongly pressor solution from the treated concentrate, but no pressor activity was found in the solution from the untreated concentrate. The pressor activity from the lead precipitate did not, however, account for six-sevenths of the *l*-nicotine added, but *l*-nicotine was shown to be fairly strongly adsorbed by freshly precipitated lead sulphide.

These results show some discrepancy between the behaviour of added *l*-nicotine and that of the base A when submitted to the processes described in § 4 for the isolation of the base A from the lead filtrate of urine concentrate.

It has also been noted that the ultra-violet absorption band at 2600 A. given by the base A, may, in crude solutions, shew very little intensification in acid solution. No explanation can be given at the present time of the minute insoluble crystals which separated from a solution of the oxalate of the base A, and which are described in § 1; and the indication, obtained in § 3, of the possible presence of a carbonyl group in the original form of the base A has not yet been confirmed. Any valid discussion of these findings must await the results of further investigation.

SUMMARY

1. A pressor base, the base A, has been isolated from the urine of humans and of bitches. It is identified in final form as *l*-nicotine.

2. Certain discrepancies are recorded, which suggest that this base may be more active than *l*-nicotine.

3. Approximately 3 cat pressor doses of the base A (a dose being the amount producing a rise in blood pressure of the order of 40 mm. Hg in the chloralosed

cat) were obtained per 1 l. of the urine of male patients and of bitches. Only one such dose was obtained per 1 l. of the urine of female surgical patients.

4. An interesting oxalate of *l*-nicotine is described.

All microanalyses, and determinations of optical activity and molecular weight were carried out by Dr G. Weiler, Oxford.

It is a pleasure to record my indebtedness to Dr F. B. Parsons for permission to collect urine from patients in Addenbrooke's Hospital, and to the Sisters in charge of the Examination Hall Hospital Extension and of Hatton, Victoria, and Musgrave Wards for their willing co-operation and assistance.

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FURTHER OBSERVATIONS ON THE EFFECTS OF ALLOXAN ON THE PANCREATIC ISLETS

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In the accounts which have been given [Dunn, McLetchie & Sheehan, 1943; Dunn, Kirkpatrick, McLetchie & Telfer, 1943] of the finding of necrosis of the pancreatic islets after intravenous injection of alloxan in rabbits, it was pointed out that this lesion came to light only because of the unexpected deaths at 12–48 hr. of certain of the animals so treated. Later it was recognized that such deaths were preceded by hypoglycaemia and hypothermia, and sometimes by convulsions as had been observed by Jacobs [1937]. The doses which gave fatal results were usually relatively large, 200–300 mg./kg. body weight, but it was known, from experiments done prior to finding the islet lesions, that similar deaths might occur, though rarely, after a much smaller dose, in one case 25 mg./kg. On the other hand, some rabbits had survived for more than 48 hr. doses of 300 and even 500 mg./kg., given on 1 day, without it being realized from ordinary observation that they had passed through a critical phase. As the pancreas was not examined in these animals the condition of the islets was not ascertained, nor was any alteration of the blood sugar suspected. Once the lesion of the islets had been found it appeared desirable that further information should be obtained as to the conditions of its occurrence and its pathogenesis, and it has been possible to carry out some experiments with this in view. These experiments have combined general observations on the animals and estimations of blood sugar with histological examination of the pancreas, and have been intended to find answers to three main questions: (1) the frequency of occurrence of islet necrosis after single intravenous doses of alloxan such as have been known to produce it; (2) the rate of development of necrosis from the earliest stages, and (3) the effects, if any, of smaller doses.

Experiments

The rabbits used were of various breeds. They were kept in metabolism cages and their usual daily food was 150 g. each of oats and bran, mixed with 200 c.c. water. About 150 g. cabbage was also given daily with rare lapses due to difficulties of supply. The output of urine was measured: in testing for sugar Benedict's reagent was used. The blood sugar was estimated by the Folin and Wu method. For fixation of all tissues, except the pancreas, Bouin's solution gave satisfactory results. For the pancreas the best results were obtained with Helly's formol-Zenker solution (5–8 hr.), but even with this fixative it proved more difficult to secure standard fixation of the soft and rather loose tissue of the rabbit pancreas, especially from freshly killed animals, than of any of the other organs. Under- and over-fixation are both undesirable and the differing thickness of the various parts of the pancreas of different rabbits may render it difficult to avoid either of these.

Stains used have been Mayer's acid haemalum and eosin, methylene-blue eosin, and modifications of Bensley's fuchsin—aniline-blue—orange method [Warren, 1938] as follows:

1.	Acid fuchsin	20 g.	
	Aniline oil	5 c.c.	
	Distilled water	100 c.c.	10 min.
2.	Wash rapidly in distilled water		
3.	1 % phosphomolybdic acid		10 min.
4.	Stain for ½–1 hr. or more in:		
	Soluble blue	0·5 g.	
	Orange G	2·0 g.	
	Oxalic acid	2·0 g.*	
	Distilled water	100 c.c.	
5.	Differentiate in methylated spirit		2–4 min.
6.	Dehydrate and mount		

* Or 8 c.c. glacial acetic acid.

Stage 4 should be continued until the acinar tissue is blue and only the α-cells and red corpuscles are strongly red. Differentiation renders normal β-cells blue to low-power examination.

The special feature of this method which has been relied upon for the present work, is its strong selective staining of α-cells in orange-red or red. This is so distinctive, even in acutely damaged islets, that cells not so stained can clearly be placed in another category and we have reckoned the latter as β-cells. In live-fixed tissue the stainable material in the α-cells may only rarely appear as distinct granules. The method renders the granules of normal β-cells blue or blue-violet, but so far this result has not been obtained with certainty in acutely damaged cells, which tend to take the orange.

(1) *Effects of single large doses of alloxan. Exps.* 1–9

The pancreas was examined in nine rabbits after single large doses of alloxan alone. During the first few hours these animals were quiet and inclined to be stationary, but were quite strong when handled and some would eat cabbage or carrot. No. 6 developed hypoglycaemic convulsions at 5¼ hr. and no. 7 at 6½ hr. and had to be resuscitated by glucose *per os*. Fig. 1 shows the movements of blood sugar in nos. 4–9 for 4–6½ hr. After the last recorded reading, glucose was given by stomach tube to all except no. 5 in order to prevent death from hypoglycaemia during the night; and this was successful except in no. 6. No. 5, which received no glucose, appeared perfectly normal until it was killed at 24 hr. Nos. 4, 6 and 8 had been given water only on the day before injection; the others were fed normally. Nos. 4 and 6, which were starved, had comparatively slight initial rises of blood sugar and developed hypoglycaemia early: no. 8 had a high initial rise but was hypoglycaemic at 6 hr. Against this, no. 7, which was fed normally, had no initial rise of blood sugar and developed hypoglycaemic convulsions at 6½ hr. Other significant data are given in Table 1. In four other rabbits which had doses of 200 mg./kg. there was glycosuria continuing for 18 days up to at least 3 months. From these results it appears justifiable to conclude that destruction of islet cells, which may be

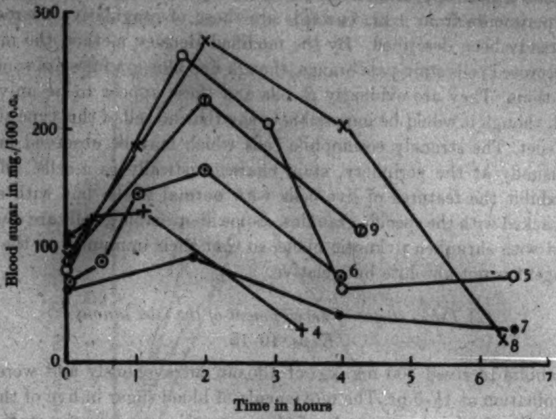


Fig. 1.

TABLE I

No.	Alloxan mg./kg.	Result	State of islets
1	300	Killed at 9 hr.	Necrosis in all
2	300	Killed at 12 hr.	Necrosis in all
3	300	Died at 18 hr.	Necrosis in all
4	300	Died at 33 hr.	Necrosis in all
5	300	Killed at 24 hr.	Necrosis in all
6	200	Died at 15 hr.	Necrosis in all
7	200	Glycosuria: killed at 25 days	Almost complete disappearance
8	200	Glycosuria: died at 10 days	Almost complete disappearance
9	200	Glycosuria 20 days: killed at 50 days	Loss of some islets and disorganization of others with regeneration

very extensive or almost complete, always results from intravenous injection of at least 200 mg./kg. of alloxan, but unless hypoglycaemic convulsions develop it is possible to have little indication from general observation that an animal is ill.

The histological appearances of damaged islet cells in the acute stages of such experiments from 9 hr. onwards are those of coagulative necrosis and have already been described. By the modified Bensley method the majority of the necrosed cells stain pale orange, though fine blue granules are sometimes seen in them. They are evidently β-cells and these appear to be universally affected, though it would be impossible to say that no cell of this type survives in any islet. The strongly eosinophile cells which may be observed in many islets, usually at the periphery, stain characteristically as α-cells. Many of these exhibit the features of live cells with normal nuclei but with swollen bodies packed with the specific granules. Some identifiable α-cells are obviously necrosed with shrunken pyknotic nuclei so that their immunity to the action of the agent is not absolute but relative.

(2) *Early stages of development of the islet lesions.*
Exps. 10–15

Six rabbits received 300 mg./kg. of alloxan intravenously and were killed for examination at 1¼–5 hr. The movements of blood sugar in five of these are shown in Fig. 2. In the accounts of these experiments only the state of the islets is described. In all cases the acinar tissue of the pancreas was normal, but eosinophile material of colloid appearance was sometimes seen in the smaller ducts. The thyroids and suprarenals showed no changes.

Exp. 10. Wt. 1900 g., killed at 1¼ hr. On low-power examination the islets had an open cribriform appearance in excess of anything seen normally: with the high power this was confirmed in that the cell ribbons were separated from one another by a space, fully the width of a cell: scanty fine basophile particles were frequently present in this space (Pl. 1, fig. 4). With haemalum and eosin the cell bodies were of normal appearance, but in the majority of the central cells, no doubt all β-cells, the nuclei were definitely hyperchromatic though retaining their normal size and outline and the appearance of a reticulum. With Bensley's stain the α-cells, which normally are relatively scanty and may be from 0 to 8 in any islet section, were of normal appearance and had normal nuclei. The β-cells showed no depletion of blue-stained material in their cytoplasm but distinct granules were not identified.

Exp. 11. Wt. 1900 g., killed at 1½ hr. The islets showed less degrees of the changes seen in Exp. 10: hyperchromasia of nuclei was less and affected fewer cells.

Exp. 12. Wt. 1940 g., killed at 2 hr. The appearances in the islets were as in Exp. 10 and not more advanced.

Exp. 13. Wt. 2000 g., killed at 3 hr. The islets showed well-marked changes: hyperchromasia of nuclei in the central cells was intense, sometimes obliterating the appearance of a reticulum, but there was no karyorrhexis. The cytoplasm of these cells stained more homogeneously with eosin than at the earlier stages or in normal cells. In addition, some cells were dislocated from attachment to their fellows, and while some of these retained their quadrate or polygonal shape a few had become rounded off and had paler stained cytoplasm. The strongly eosinophile α-cells were quite unusually prominent, most being peripheral but some central in the islets. In the majority of the α-cells the nuclei were normal, but in a few, with swollen rounded bodies, the nuclei were shrunken and pyknotic. Bensley's stain gave much more definitive appearances. The brightly stained α-cells were swollen to twice their normal size or more, and had usually become rounded in shape. They appeared more numerous than normally and sometimes formed an almost continuous ring of

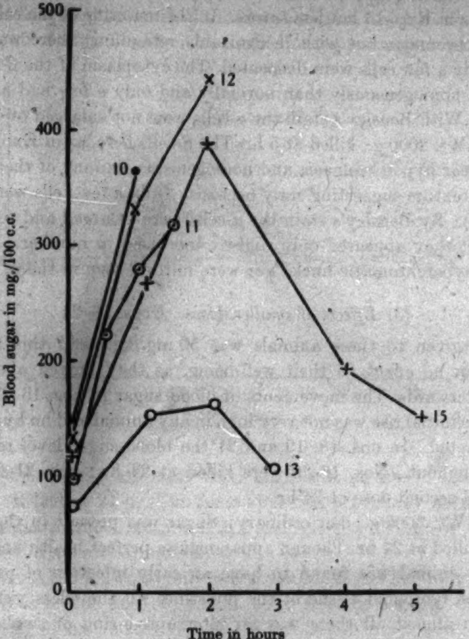


Fig. 2.

up to a dozen cells at the periphery of an islet. The appearance of increase in number was difficult to account for except by the actual increase in size: no mitoses were seen in them. The bodies of the β-cells mostly stained heavily and tended to retain some of the orange; granules could not be seen. Some of the dislocated cells had a washed-out appearance with some granular substance remaining, but it could not be decided that this was specific granulation.

Exp. 14. Wt. 1900 g., killed at 3 hr. 40 min. The islet changes were of the same kind as in Exp. 13 but less severe. In the majority of the cells the nuclei were hyperchromatic but with recognizable reticulum: there was no karyorrhexis. Only a few cells were dislocated. The cytoplasm of the β-cells stained rather more homogeneously than normally and only a few had a washed-out appearance. With Bensley's stain the α-cells were not enlarged or conspicuous.

Exp. 15. Wt. 2000 g., killed at 5 hr. The β-cells here, as in Exp. 13, showed intense nuclear hyperchromasia and homogeneous staining of their cytoplasm with loss of texture suggesting early necrosis. Only a few cells were dislocated (Pl. 1, fig. 5). By Bensley's stain the α-cells were enlarged and rounded as in Exp. 13, but they appeared only slightly increased in number. None of the α-cells had hyperchromatic nuclei nor were mitoses seen in them.

(3) *Effects of smaller doses. Exps.* 16–24

The dose given to these animals was 50 mg./kg., and this appeared to have little or no effect on their well-being, as they moved about and fed normally afterwards. The movements of blood sugar in nos. 16–23 are shown in Fig. 3. The initial rise was not very high in any animal and no hypoglycaemic phase was noted. In nos. 18, 19 and 21 the blood-sugar level remained unaltered throughout. Nos. 16–20 were killed at 24 hr.; nos. 21–24 at 48 hr. No. 24 had a second dose at 24 hr.

Exp. 16. Wt. 3000 g., diet ordinary. Sugar was present in the urine next morning. Killed at 24 hr. Though apparently in perfect health and eating full rations, this animal was found to have an early infection of pseudo-tuberculosis in the lymphoid tissue of the intestine. All the islets were markedly changed. In almost all there was an interrupted ring of swollen, brightly eosinophile cells at the periphery with sometimes a few centrally. These were identifiable as α-cells by Bensley's stain. In the centres the β-cells were almost all rounded off and slightly swollen: their nuclei were pyknotic, yet few showed karyorrhexis or karyolysis: their bodies generally stained pale with eosin and in some the stainable substance appeared washed-out (Pl. 1, fig. 6). With Bensley's stain fine granules could be identified in the β-cells, usually numerous, but in some, corresponding to those described as washed out, they were very scanty, giving the cell bodies an empty appearance.

Exp. 17. Wt. 2300 g., water only was given for 24 hr. before injection. The blood sugar rose to 230 mg./100 c.c. at 4½ hr. Glucose 5 g./kg. was given by stomach tube at 4½ and at 6½ hr. There was slight glycosuria in the morning. Killed at 24 hr. All the islets showed changes of the same kind as in Exp. 16, though somewhat less in degree.

Exp. 18. Wt. 1350 g., received water only for 24 hr. before injection. There was no rise of blood sugar at 2 or at 4 hr., and no glycosuria appeared. Killed at 24 hr. Many of the islets here retained normal appearances. It was only on systematic examination with the high power that any changes were recognized. These affected only about a tenth of the islets and were restricted to dislocation and rounding off of a few β-cells with a washed-out appearance in some of these.

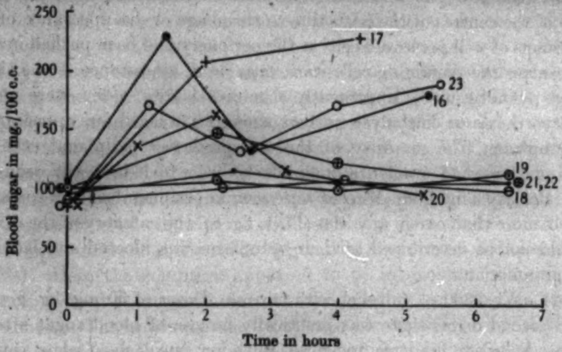


Fig. 3.

Exp. 19. Wt. 1500 g., ordinary diet. There was no rise of blood sugar at 2 or at 4 hr. Killed at 24 hr. The islets appeared practically normal. One or two hyperchromatic nuclei were seen in a few, but it could not be decided that this constituted a definite abnormality.

Exp. 20. Wt. 1150 g., water only was given for 24 hr. before injection. The blood sugar rose to 165 mg./100 c.c. at 2 hr. Glucose 3 g./kg. was given by stomach tube at 6½ hr. and 2 g./kg. at 11½ hr. Sugar was present in the urine next morning and the blood sugar was 320 mg./100 c.c. Killed at 24 hr. Bouin fixation only was available. The majority of the islets appeared normal but in a few there was a washed-out appearance of some of the central cells.

Exp. 21. Wt. 1600 g., water only was given for 24 hr. before injection. The blood sugar rose from 99 to 147 mg./100 c.c. at 2 hr. No glycosuria. Killed at 48 hr. The majority of the islets appeared quite normal even on close inspection, but in one or two there was a group of about a dozen washed-out and crumpled cells with pyknotic nuclei in the centre. Similar appearances affecting fewer cells could be recognized in other islets. There was only a slight degree of swelling and prominence of α-cells in some islets.

Exp. 22. Wt. 1600 g., ordinary diet. There was no rise of blood sugar at 2 hr. Killed at 48 hr. The islets generally appeared normal but on close examination with the high power one or two dislocated cells with shrunken bodies and pyknotic nuclei were seen in a few of them. No mitoses were found.

Exp. 23. Wt. 1150 g., ordinary diet. The blood sugar rose from 98 to 170 mg./100 c.c. at ½ hr. and remained at about that level for 5½ hr., falling to 110 mg. at 10 hr. Next morning it was 120 mg. The animal was killed at 48 hr. On low-power examination the islets were notably altered, the majority being reduced in size and having very irregular ill-defined outlines (Pl. 1, fig. 7). With the high power it was clear that this deformation had resulted from collapse of the centres of the islets due to shrinkage or disappearance of cells, whilst groups of well-preserved cells at the periphery had been pushed inwards. In the centres the remaining cells were variable in appearance: some showed shrunken pyknotic nuclei and greatly shrunken bodies: a few were necrotic with karyorrhexis or karyolysis: others showed a washed-out appearance of their cytoplasm. The majority of the well-preserved peripheral cells were α-cells and these had swollen eosinophile bodies which took the orange-red stain by Bensley's method. Mitoses were seen in peripheral cells in some islets but rarely more than one in any islet (Pl. 1, fig. 8): the category of the dividing cells could not be determined as their cytoplasm was bloated and lacking in specific granulation.

Exp. 24. Wt. 2300 g., full diet with carrots. Doses of 50 mg./kg. given on first and second days: there was practically no rise of blood sugar after the first dose. Killed 48 hr. after first dose. With low power most islets appeared normal but the high power revealed various degrees of damage in about one-fifth: the commonest change was dislocation and rounding off of β-cells; their nuclei were hyperchromatic and the cytoplasm had a washed-out appearance (Pl. 1, fig. 9). In one or two islets there was definite necrosis of a central mass of cells. The α-cells in damaged islets were generally swollen and prominent. No mitoses were found on careful search either in the damaged islets or in the more normal ones.

DISCUSSION

The results of the first nine experiments, and of four others referred to, show that intravenous doses of alloxan of at least 200 mg./kg. in rabbits always cause severe damage to the islets of Langerhans. While this lesion is developing most animals are somewhat depressed and may not eat, and some pass into hypoglycaemic convulsions after 4–24 hr.: death then occurs unless glucose is administered [cf. Jacobs, 1937]. It is possible, however, for a rabbit to survive a dose of 200 mg./kg. for 24 hr. with necrosis in the whole of its islets and without showing very definite outward signs of illness (Exp. 5). This fact, which could hardly have been expected where such a specialized and important tissue was concerned, helps to account for the apparent capriciousness in effect even of large doses of alloxan; it may also have some significance in relation to the possible occurrence of acute damage of the islets from any cause in the human subject.

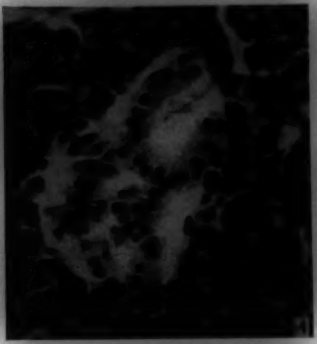
It has already been shown that with large doses of the above order the islet cells exhibit the features of coagulative necrosis at nine hours, and the view that by that time they are dead is confirmed by the progress of nucleolysis in animals examined at later periods. In Exps. 10–15 the doses were 300 mg./kg., and it is considered certain that frank necrosis would have been recognizable in the islets had the animals been allowed to live for 9 hr. or longer. Histological examination revealed certain changes from 1¼ hr. onwards: the earliest and most definite was hyperchromasia of β-cell nuclei, which preceded any recognizable cytoplasmic change. The hyperchromasia was increased in intensity at 3 and at 5 hr. and was then associated with altered staining of the cytoplasm and with dislocation of some cells. It is impossible to tell by histological examination the earliest stage at which cells are dead, but it seems probable that death had occurred in some cells at 3 hr. when they were separating from their attachments: it may, of course, have occurred earlier. The opening up of channels between the cell ribbons (Pl. 1, figs. 4, 5) at these early stages cannot be interpreted with certainty. Some degree of this may be seen at times in normal islets, though usually the ribbons are closely packed together. Here the condition appeared to be in excess of normal and the spaces contained scanty granular debris suggesting that they had had some fluid content. The appearance is thought to be compatible with a temporary increase in functional activity of the islets which might be in operation immediately after dosage.

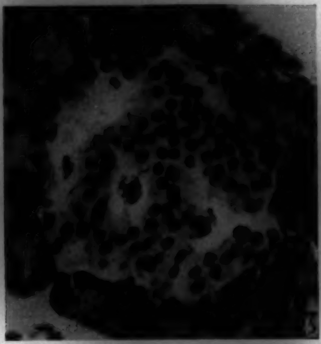
From observation of the general behaviour only of rabbits which received 50 mg./kg. of alloxan (Exps. 16–24), it would not have been suspected that any serious internal lesion had occurred. Examination of the pancreas in these animals gave variable results. In one (no. 19) all the islets appeared normal; in four, nos. 18 and 20 killed at 24 hr., and nos. 21 and 22 killed at 48 hr., the majority were normal and only slight changes were present in the remaining few: these comprised nuclear pyknosis and depletion of cytoplasmic substance in some cells, resulting in a washed-out appearance by ordinary staining. The focal distribution of these slight lesions, notable also in Exp. 24, may possibly be related to the state of activity of individual islets during the period when the agent was having effect. In contrast with these slight changes, there were quite pronounced alterations in the islets in nos. 16 and 17, examined at 24 hr., and in no. 23, examined at 48 hr. In these the β-cells were much changed: they were rarely frankly necrotic but their outlines were rounded off and their nuclei hyperchromatic, while the granular substance in their cytoplasm was much depleted. With the special stain great diminution of the fine β-granules was notable in many cells. By 48 hr. most of these cells had shrivelled or collapsed, taking up little space, so that the islets were small with deformed outlines. These cellular changes were markedly in contrast with those of coagulative necrosis and karyolysis seen at the same periods after large doses, and as they had taken so long to develop it appeared to be a reasonable interpretation that while essentially necrobiotic in character, they were the result of overactivity and exhaustion of the cells after stimulation, rather than of a directly lethal toxic effect.

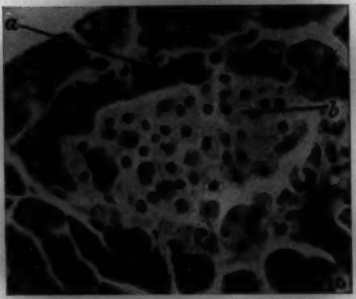
A further interesting and highly suggestive feature in the histological appearances in the islets is the differential effect on α- and β-cells. It had already been noted that in islets necrosed after large doses any surviving cells were situated marginally, and that their bodies were strongly eosinophile. It is now confirmed by special staining that these are α-cells and that their bodies are enlarged to two or three times normal, with evident increase in amount of the specific granule substance. This change may be well established 3 hr. after a large dose and is also quite pronounced 24 hr. after a smaller dose wherever the β-cells are much altered. With this change, which appears to be an active and progressive one, the majority of the α-cells have the appearance of living cells, but some are necrosed. It seems likely that this striking difference in their behaviour is significant of some considerable difference in the respective functions of α- and β-cells, which by this method may become accessible to investigation.

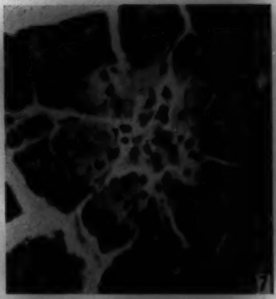
Although the movements of the blood sugar were observed only over the first few hours, a correlation of them with histological changes provides some interesting features. In eleven animals which received large doses (Figs. 1, 2) there was a rise of blood sugar in 2 hr. to 230–440 mg./100 c.c. in seven; and to 130–165 mg. in three, two of which had been starved for 24 hr. In one only, no. 7, which was fed normally, the blood sugar did not rise to 100 mg./100 c.c., but in this animal the initial reading was unusually low and hypoglycaemia set in rapidly. After doses of 50 mg./kg. the blood sugar rose in only five out of eight and the elevation was less than with the large doses: of these five, three were those with well-marked changes in the islets.

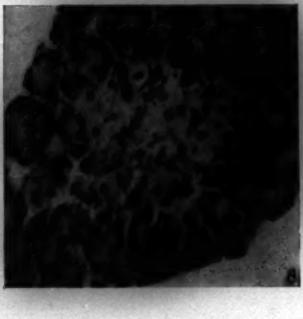
From these observations it may be concluded that a definite initial rise of blood sugar after alloxan probably indicates that damage of islets is occurring. Absence of a rise may mean that damage is slight or nil, but may also be noted even if this is severe. Jacobs, in referring to the transient hyperglycaemia, did not conclude that it was a specific effect of alloxan. The hypoglycaemia which may be evident in 4 hr. appears to be a constant and specific effect if the dose of alloxan is sufficient. Jacobs observed it invariably with doses of 70 mg./kg. and upwards, and we can confirm this from all experiments where the dose was sufficient and suitable examination was made. It will be noted that no hypoglycaemia was observed after doses of 50 mg./kg. (Exps. 16–24).

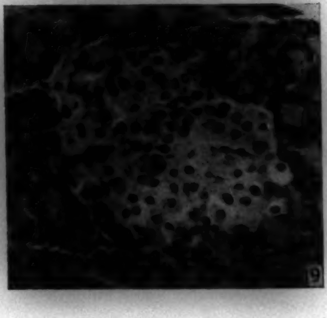












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SUMMARY

1. From observations on fourteen rabbits it has been confirmed that single intravenous doses of alloxan of at least 200 mg./kg. always cause serious damage of the islets of Langerhans, though there may, during the first day, be little outward indication of serious illness. After these large doses definite histological changes can be recognized in the islets after about an hour, and these may be very pronounced at 3 hr. when some β-cells appear to be necrosed.

2. With doses of 50 mg./kg. the β-cells sometimes show degranulation, but little necrosis, at 24 hr.: the appearances are consistent with abnormal increase of function. After these smaller doses islet changes may be only slight and focal in distribution.

3. α-cells are less prone to the necrobiotic changes than β-cells and, from early stages, may be enlarged with apparent increase of granules when the β-cells are necrosed or exhausted: this observation suggests some difference in function of the two types of cell.

4. A temporary hyperglycaemia almost always occurs in the first 2 hr. where there is any damage to the islets.

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EXPLANATION OF PLATE 1

Fig. 4. Exp. 10. Hyperchromasia of nuclei and opening of spaces between cell ribbons. Dose 300 mg./kg.: time 1¼ hr. H. and E. ×400.

Fig. 5. Exp. 15. More intense nuclear hyperchromasia, dislocation of some cells, and spaces formed. Dose 300 mg./kg.: time 5 hr. H. and E. ×480.

Fig. 6. Exp. 16. Rounding off of β-cells with nuclear hyperchromasia and pale staining of cytoplasm: washed-out appearance in some cells (*b*). A border of enlarged α-cells above with normal nuclei and strongly stained bodies (*a*). Dose 50 mg./kg.: time 24 hr. H. and E. ×480.

Fig. 7. Exp. 23. Collapsed and deformed islet with disappearance of most of the central β-cells: clumps of large α-cells peripherally. Dose 50 mg./kg.: time 48 hr. H. and E. ×280.

Fig. 8. Exp. 23. Deformed islet, mainly α-cells. Mitosis is seen in a cell at the upper edge. H. and E. ×280.

Fig. 9. Exp. 24. Nuclear pyknosis and pale cytoplasmic staining of β-cells in lower part of islet, with washed-out appearance in some. Normal cells in upper two-thirds. Dose 50 mg./kg. first and second days: killed at 48 hr. H. and E. ×280.

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HYPERPNOEA IN MAN PRODUCED BY SUDDEN RELEASE OF OCCLUDED BLOOD

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In the course of some experiments on human subjects in which venous congestion was produced by the inflation of a Riva-Rocci cuff to between arterial and venous pressures, it was observed that a brisk hyperpnoea often followed the removal of the cuff. The hyperpnoea occurred so promptly that it appeared unlikely to be due to the arrival of blood with high CO_2 and low O_2 content at the chemoreceptors or respiratory centre. The origin of this hyperpnoea seemed worthy of further investigation. An attempt was therefore made to find the point in the circulation reached by the released blood when hyperpnoea began. Since there are no known chemoreceptors in the pulmonary vascular bed, a hyperpnoea initiated in this portion of the circulation must arise from pressure receptors.

METHODS

The subjects used were the author and a laboratory assistant, both accustomed to respiratory experiments, and a number of male medical students. All, so far as is known, were in perfect health.

Respiration was recorded with a chest stethograph and an ink writer on a vertical drum, normally moving at about 5 mm./sec. The subject lay on a surgical couch with the head and shoulders slightly raised, and Riva-Rocci cuffs were applied to one or two limbs at pressure of 60–90 mm. Hg for about 10 min. The observer then abruptly removed the cuff and simultaneously pressed a signal marker, and the interval which elapsed before the ensuing hyperpnoea was subsequently measured on the paper. In some experiments the magnitude of the initial hyperpnoea was measured by collecting in a small rubber bag the two first expirations of the hyperpnoea, timing the collection over two complete respiratory cycles, and measuring the volume in a wet gas meter. The magnitude of the ventilation was calculated in l./min. reduced to S.T.P. dry.

In order to determine the point in the vascular system reached by the released blood when hyperpnoea commenced, ether or sodium cyanide was sometimes injected into a vein just distal to the occluding cuff immediately before release. The site of the injection was anaesthetized with procaine to avoid respiratory disturbance from the pain of the injection; nevertheless, when ether was injected considerable pain was often felt, which sometimes prevented the injection of the intended amount. It appeared that pain was aroused by prolonged contact of ether with the intima of the vein, and it was best avoided by a very rapid injection. It was found necessary to inflate the cuff to over 150 mm. Hg just before the injection, since at lower pressures the subject smelt ether before removal of the cuff. The amount of ether used was 1·8–4 c.c. of a 4 or 8% solution in isotonic saline, different subjects varying in the ease with which they could smell it. The moment when the smell was perceived was indicated by the subject by pressing a signal marker, and a few experiments in which this point was not sharp were discarded; apart from these, the dosage of ether did not appear to affect the latency of the response. Sodium cyanide was injected in amounts of 0·5–0·7 c.c. of a 2% solution.

Robb & Weiss [1933] have demonstrated that a prompt hyperpnoea signals the arrival of cyanide at the carotid body. Subjects who could smell it signalled the time as with ether.

RESULTS

Thirteen subjects have been tested, and of these, two showed no hyperpnoea on release of venous occlusion, whilst the other eleven all under some circumstances showed hyperpnoea within at most 4·2 sec. The form of the respiratory tracing in different subjects varied considerably, some taking a series of deep, rather slow breaths, others rapid shallow ones, some just taking a single large breath, whilst in a few there was a series of rapid, deep breaths. The one appearance common to all the tracings which showed any change at all was a steepening of the inspiratory slope; the stimulus seems in fact to be an inspiratory one. The subjective sensations experienced by different subjects at the onset of the hyperpnoea included a feeling of constriction in the lower chest and epigastrium and throbbing in the head.

After perhaps 10 sec. the hyperpnoea may be influenced by the chemical effect of asphyxial blood. One subject in fact showed a very small initial hyperpnoea, which after 15 sec. changed into a spell of very rapid breathing which persisted for 1½ or 2 min.

In most experiments congestion was applied for 10–15 min.; but in the three subjects who showed the best hyperpnoeic response the magnitude of the initial hyperpnoea, i.e. the first two hyperpnoeic breaths, was measured after varying periods of congestion. This showed a very large random variation in any one subject, as well as considerable difference between subjects. In J.N.M. it ranged between 19·1 and 92·4 l./min. and in N.C.H.J. between 6·7 and 15·6 l./min. There was no apparent correlation between the time for which congestion was applied and either the latency or the magnitude of the response, provided that congestion was applied for more than 4 min.; with shorter periods there was often no hyperpnoea, and occasionally the same was true with longer periods, especially if the subject had only just lain down before the experiment began. The lack of an increased effectiveness of long periods of congestion is evidence against a chemical stimulus contributing to the initial hyperpnoea.

The hyperpnoeic response is somewhat dependent upon posture. Two subjects showed hardly any effect if, instead of lying supine, they sat upright in a chair, but one showed as good a response in this position as when recumbent.

On one occasion the experiment was performed with a cyanide injection with the subject lying on his side. The hyperpnoeic response was vigorous, and uncomfortable periodic breathing ensued. This was not diminished by turning the subject on to his back, nor by giving him oxygen to breathe for two periods of 70 and 80 sec., nor by carbon dioxide. It ceased promptly when, 20 min. after its onset, the back of the couch was raised to 45°. Periodic breathing has also occurred when a subject was lying on his back throughout and when no cyanide was given. It has sometimes subsided spontaneously, but if it persisted oxygen never abolished it, whilst raising the back decreased or abolished it. The initial hyperpnoea was sometimes so vigorous as to produce paraesthesia, so that a subsequent periodicity could be explained on the classical chemical theory of Douglas & Haldane [1909], were it not that oxygen does not affect it.

Ether. In two subjects the time intervals after removal of the cuff until the onset of hyperpnoea and the appreciation of the smell of ether have been repeatedly determined and the mean results with the standard errors of these means are shown in Table 1. It will be observed that the hyperpnoea precedes the smell by 1 or 2 sec.

TABLE 1. Time in sec. after removing cuff until hyperpnoea occurs and ether is smelt.
(Full description in text.)

Subject ...	J.N.M.	T.J.M.
Mean time to hyperpnoea	2·2 ± 0·23 (11)	1·6 ± 0·36 (8)
Mean time to smell	4·4 ± 0·33 (9)	2·9 ± 0·37 (7)
Mean difference	2·1 ± 0·24 (9)	1·7 ± 0·63 (6)

Figures in parentheses indicate the number of experiments from which each mean was derived.

It has commonly been assumed, as by Miller [1934], that the time after an ether injection until it is smelt represents the vein-lung circulation time; but there must be a systematic error in this assumption representing the reaction time between the arrival of an adequate ether concentration at the olfactory endings and the motor response of the subject, together with an error due to the time taken for an effective concentration of ether to pass from the lungs to the olfactory endings which will probably vary with the phase of respiration at which ether arrives at the lungs. Such errors may be trifling under the circumstances in which ether circulation time is usually determined, but may well be important when the venous return is suddenly accelerated by release of a congesting pressure. An attempt was therefore made to determine their magnitude.

The subject with a nose clip breathed through a mouthpiece and a wide-bore two-way tap whereby he could be suddenly connected to a tube containing cotton-wool soaked in a saturated solution of ether in normal saline, the whole being immersed in a water-bath at 37° C. and the dead-space being about equal to the respiratory dead-space. It had first been found that ether was much more readily 'smelt' if admitted to the mouth than to the nose. On the same two subjects, the mean time between turning the tap and the depression of a key by the subject to indicate that he had smelt ether was 2·57 sec. for J.N.M. and 2·10 sec. for T.J.M. These means are derived from seventeen and twenty-three experiments respectively, in which the time of turning of the tap was randomly distributed between all phases of respiration. The interval was longer if the tap was turned early in expiration, and it appeared that ether was not smelt until inspiration commenced. However, in those experiments in which ether was injected, it was sometimes smelt towards the end of inspiration. In comparing the time interval to smelling ether after addition to the inspired air or after injection I therefore neglected the respiratory phase, assuming in both sets of experiments a random distribution throughout the different phases.

It appears from the figures given that the difference between the time of onset of hyperpnoea and the time of smelling of ether in the experiments referred to in Table 1 can be more than accounted for by ether diffusion time and the subject's reaction time; but a statistical comparison is desirable. The 'mean difference' in Table 1, *d*, is a statistically more reliable figure than the difference between the two means, and this may be compared statistically with the mean time interval, *i*, between the subject's exposure to ether vapour and his pressing a key to indicate that he has smelt the ether. The difference between these quantities, *i–d*, is an estimate of the time elapsing between the arrival of blood suddenly released from a limb in the pulmonary capillaries, and the onset of hyperpnoea. Since both figures are means of statistically small samples, the small-sample technique of Fisher [1941], involving a pooled estimate of variance, was used. For J.N.M. the mean difference (*i–d*) was 0·43 sec., and its limits for different degrees of probability, calculated from Fisher's Table of *t*, are shown in Table 2. For the determinations on T.J.M. the mean of (*i–d*) was almost identical, 0·37 sec., but the random error was much greater.

TABLE 2. Extreme values (in sec.) of (*i–d*) for different levels of probability

P	Minimal value	Maximal value
0·05	−0·41	+1·26
0·02	−0·58	+1·43
0·01	−0·70	+1·55

Apart from the statistically estimable errors there may be a systematic error in the above calculations, if the time taken for an effective concentration of ether to reach the olfactory endings is not strictly comparable in the two sets of experiments. It appears to be more rapid if ether is injected, since ether can be smelt at any phase of respiration, and it may also be accelerated by hyperpnoea. Such an error would make the estimate of (*i–d*) too high, or in other words would mean that blood had not got as far in the pulmonary circuit as the figures indicate when hyperpnoea occurred. It seems in fact that hyperpnoea is almost contemporaneous with the arrival of ether in the pulmonary capillaries, but it may occur a second or two earlier or later.

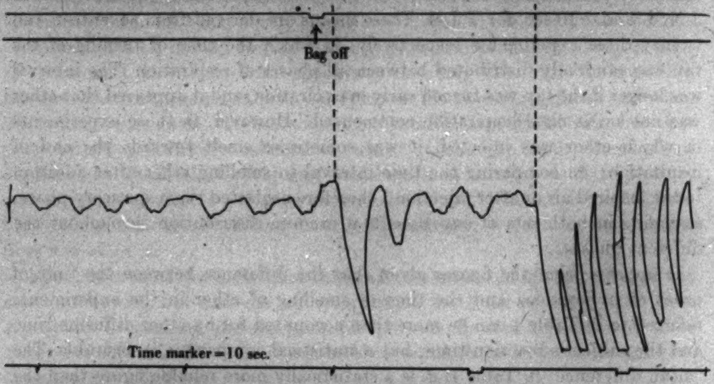

Fig. 1. Stethograph record of respiration after release from an arm of blood occluded there and containing cyanide. Inspiration downwards. Discontinuous lines are construction lines for measuring the time intervals.

Cyanide. When cyanide was injected into a vein just distal to the cuff immediately before its removal, the hyperpnoea was always diphasic, as illustrated in Fig. 1. Sometimes the first hyperpnoea was subsiding before the second appeared, as in the instance reproduced, whilst sometimes the second phase was a sudden intensification of an original sustained hyperpnoea. It appears from comparison with the figures of Robb & Weiss [1933] that the second hyperpnoea corresponds to the time of arrival of cyanide at the chemoreceptors, and this is further proof that the initial hyperpnoea is not a chemical effect of asphyxial blood. Two of the subjects used could smell cyanide, and the moment when they smelt it was recorded. Table 3 shows the time intervals found, and it will be seen that as with ether the initial hyperpnoea precedes the smell. In order to determine whether in J.N.M., who could not smell cyanide, the time interval between the two spells of hyperpnoea approximately

TABLE 3. Interval (in sec.) to hyperpnoea after releasing venous occlusion from a limb into whose vein cyanide has been injected

J.N.M.		T.J.M.		
1st hyperpnoea	2nd hyperpnoea	1st hyperpnoea	Smell	2nd hyperpnoea
1·4	8·1	1·7	4·9	11·3
1·7	15·3	1·2	3·9	9·9
2·2	14·2		N.C.H.J.	
1·8	11·2	1·7	5·0	9·7
J.M.H.B.		2·4	6·3	11·0
2·0	12·9			

represented the time taken for blood to pass from lungs to carotid body, a few determinations were made of the combined ether-cyanide circulation time without venous occlusion. The mean interval between smell of ether and hyperpnoea was 6·3 ± 0·53 sec. (S.E. of mean of five observations). Since, in experiments in which ether is injected into an occluded vein before release, the smell corresponds to a time about 2 sec. later than the hyperpnoea, it will be seen that the intervals between the two hyperpnoeic bursts shown in Table 3 roughly correspond to the time taken for blood to pass from the pulmonary capillaries to the carotid sinus. This approximate correspondence also indicates that the volume of blood released from the occluded limb is insufficient to accelerate the circulation considerably after its addition to the general venous return in the right auricle.

Blood pressure. The blood pressure was measured in the brachial artery with a sphygmomanometer on several occasions before, during and after the period of occlusion.

The only change observed was a fall of 6–14 mm. Hg in the diastolic pressure during the first minute after release of congestion, followed by a return to the previous level in about 2 min. A similar fall was produced by a brief spell of voluntary hyperpnoea about equal to the involuntary hyperpnoea which follows release of congestion. If after release of congestion the respiration was deliberately kept down to a normal level the change of blood pressure did not occur until the subject was unable to restrain the compulsion to overbreathe, when the diastolic pressure promptly fell. The fall is therefore due not to the release of congestion but to the consequent hyperpnoea.

In all instances the congesting pressure was found to be about or somewhat above the diastolic arterial pressure.

DISCUSSION

In human experiments upon a function under volitional control the possibility of psychological influences must be considered. Most of the subjects were unaware of the nature or purpose of the experiments, so that such factors can hardly be significant; whilst the two principal subjects were well accustomed to respiratory experiments and to leaving their respiration free from voluntary control. Irregular and atypical results were sometimes obtained in the first experiments with any new technique, when the subject was paying considerable attention to the procedure and was often concerned that the observer should perform his various operations correctly. When the technique was familiar and the subject could remove his attention completely from his surroundings his respiratory responses became regular and reproducible; the subject was often in fact nearly asleep and could not remember afterwards what experiments had been performed, beyond that he was aroused periodically by a sudden compulsion to inspire. This complete passivity could not of course be attained in the experiments with ether injection, but there was seldom any doubt in the mind of the subject when he suddenly felt the compulsion to inspire, and on rare occasions when there was any doubt the experiment was discarded.

Sensations will of course be aroused from the arm on release of the cuff, and somatic sensations, particularly pain, can affect respiration. In fact, however, occlusion at the low pressures used caused very little sensory disturbance and usually no pain excepting when it was applied for long periods, and long periods were no more effective than short ones in producing hyperpnoea, whilst larger painful stimuli caused less respiratory disturbance. Subjectively the hyperpnoea was associated with a vaguely defined sensation in the thorax or epigastrium, and never with sensation in the arm, whereas a gasp produced by pain is very definitely associated with the pain in the mind of the subject. It thus appears that the hyperpnoea observed was a result of the sudden release of blood into the circulation.

Harrison, Harrison, Calhoun & Marsh [1932] have described in dogs a hyperpnoea produced by rapid intravenous infusions or by inflation of a balloon in the right auricle, and prevented by vagal section, and Yeomans, Porter & Swank [1943] confirm the respiratory acceleration produced in dogs by intravenous infusions. Daly, Ludány, Todd & Verney [1937], also working with dogs, found that with the pulmonary and systemic circuits separately perfused an increase of pulmonary flow caused increased inspiratory tone and respiratory acceleration, disappearing after section of the cervical vago-sympathetic. Churchill & Cope [1929] have produced a similar respiratory acceleration by raising the blood pressure in a lung isolated from the rest of the circulation; and Sutton & Lueth [1930] describe hyperpnoea as resulting from distension of the aorta. Partridge [1939] has demonstrated that the vagus contains fibres distinct from those responding to pulmonary inflation, conducting with a cardiac rhythm, whose stimulation produces respiratory acceleration, and Hammouda, Samaan & Wilson [1943] have demonstrated that these fibres are contained in pulmonary branches of the vagus. This conception of dyspnoea arising reflexly from receptors in or near the pulmonary vascular bed is borne out by observations of the respiratory effects of multiple pulmonary embolism and their dependence upon an intact vagus, as of Dunn [1920], as well as by numerous clinical observations of the association between dyspnoea and pulmonary congestion [Weiss & Robb, 1933; Christie, 1938; Kountz, Smith & Wright, 1942].

The evidence here presented is insufficient to determine the precise site of origin of the reflex hyperpnoea described, though the moment of origin of the hyperpnoea is very close to the time of arrival of blood in the pulmonary capillaries. The absence of dyspnoea in human cases of primary sclerosis of the pulmonary arteries [East, 1940; Brenner, 1935] suggests that receptors proximal to the pulmonary arteries are unimportant as a source of dyspnoea in man.

Both by subjective observation and by inspection of the respiratory tracings the primary stimulus appears to be an inspiratory one. On different occasions it arose at different phases of the respiratory cycle, a sudden compulsion to inspire deeply occurring sometimes at mid-expiration or even at the height of inspiration, or during the course of a quiet inspiration; the stimulus would thus appear to be independent of the pulmonary stretch receptors, since it can suddenly become prepotent in any condition of lung inflation or collapse. Mackay [1943] describes a sensation of suffocation when blood occluded in the legs of human subjects is released into the circulation, but does not record whether this sensation was accompanied by hyperpnoea.

The subsequent periodicity, abolished by raising the back but not by oxygen, has suggestive similarities to some clinical forms of periodic breathing, but particularly to the orthopnoea and paroxysmal dyspnoea of some victims of cardiac disease. This dependence upon posture of a periodicity unaffected by oxygen has been observed also when breathing against a resistance, a subject who was periodic when recumbent breathing regularly when his back was raised, and in other circumstances which will be the subject of a later communication.

Haldane, Meakins & Priestley [1919] similarly found that the periodicity induced by mechanical restriction of respiration is more marked when lying than when sitting. They state that this periodicity is abolished by oxygen, but in the only tracings which they reproduce a periodicity is still discernible when the subject is breathing oxygen.

SUMMARY

1. The sudden release of blood occluded in the veins of a limb produced in eleven out of thirteen human subjects a brief hyperpnoea, which in four of these was of considerable magnitude.

2. If ether was injected into a vein just distal to the point of occlusion, immediately before release, it was smelt somewhat after the onset of hyperpnoea.

3. If cyanide was similarly injected, there was a diphasic hyperpnoea and the interval between the two phases approximately corresponded to the lung-carotid body circulation time.

4. It is concluded that hyperpnoea arose almost contemporaneously with the arrival of the blood in the pulmonary capillaries. It is suggested that it was due to a reflex from pressoreceptors in the pulmonary vascular bed.

5. The similarity to other conditions of dyspnoea associated with pulmonary congestion is discussed.

My thanks are due to all those who have acted as subjects, and especially to Mr T. J. Meadows, who has also given me technical assistance.

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INVESTIGATIONS ON MUSCLE ATROPHIES ARISING FROM DISUSE AND TENOTOMY

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It is well known that, when striated muscle is deprived of its innervation, it undergoes rapid atrophy [Tower, 1939]. Moreover, even though its motor innervation is preserved intact, a striated muscle undergoes a striking atrophy when it remains completely inactive for a period of weeks. For example, Tower [1937] completely isolated a segment of the dog's spinal cord from incoming impulses by cutting its dorsal roots and sectioning the cord above and below. The muscles innervated from this segment remained completely inactive and showed a rapid and progressive wasting.

Disuse atrophy has been further studied in cats, using somewhat similar preparations [Eccles, 1941]. The lumbar and sacral regions of the spinal cord were cut off from all incoming nerve impulses by an initial operation in which the lumbar and sacral dorsal roots on both sides were severed and the cord transected just above the first lumbar segment. After 3 week's disuse the muscle weights had declined to about 60% of normal and the contraction strengths had fallen still further. With two muscles (the ankle flexors, extensor digitorum longus and tibialis anticus, henceforth termed E.D.L. and T.A.) it was further shown that daily intermittent tetanization for any duration between 10 sec. and 2 hr. practically prevented the loss of weight. However, the ratios

$$\frac{\text{maximum tetanic contraction}}{\text{muscle weight}} \text{ and } \frac{\text{maximum tetanic contraction}}{\text{maximum twitch contraction}},$$

which fell in consequence of the disuse, showed but a small increase towards normal as a result of the intermittent tetanization. The other two muscles (the ankle extensors, soleus and the medial head of gastrocnemius) were still less affected by the daily tetanization, there being some prevention of weight loss but otherwise no significant improvement. It was pointed out that the differences between the flexor and extensor groups of muscles may be due to the different conditions under which the muscles contracted. Since the ankle remained fully extended, both during the ¼ sec. periods of stimulation and the ¾ sec. intermissions, the flexors were maintained fully lengthened and the extensors fully shortened. With both groups the experimental stimulation further differed from the normal activity of muscles in that it did not reproduce the range nor the alternation of lengthening and shortening which occur in natural movements.

The present experiments were designed to test these possibilities. In most experiments either the flexor or extensor group of muscles was tenotomized. The atrophy of tenotomized muscle was thus incidentally investigated, and proved to be of such interest that it was further studied.

METHOD

In the present series of thirty cats, inactivation of the muscles was brought about by the operation described above. All but two animals survived this initial operation and remained in good condition until the final investigation 21 or 22 days later. The average weight loss of the cats was 7% of the pre-operative weight. The greatest loss was about 20%, while several regained their pre-operative weight. This average weight loss is no more than would be expected from the disuse atrophy of the lower trunk and hind limb muscles, i.e. as regards general condition the cats remained practically normal.

For experiments involving muscle shortening or lengthening during stimulation it was necessary to tenotomize either the ankle extensors or flexors. Of the extensors, the whole tendo Achilles was divided at its insertion into the calcaneus and the others were also tenotomized at the ankle. The tendons of the two flexors, T.A. and E.D.L., were severed at least 1 cm. beyond the muscle attachment and the peroneal tendons were also divided.

The daily tetanizations of the muscle were provided by maximal stimulation of the sciatic nerve through the skin by means of a localized pad over the nerve and a large indifferent pad. The frequency of stimulation was about 40 per sec., ¼ sec. periods of stimulation alternating with ¾ sec. intermissions. After every 15 sec. of stimulation there was a period of rest for 15 sec. Total daily periods of stimulation were constant for any one experiment, but durations of 30 sec., 2 min. and 4 min. were used in different experiments.

In all experiments, except one, the muscles exhibited no spontaneous movements at any time after the operation. In the exceptional case the muscles exhibited occasional brief movements which, as described by Tower [1937], may have been due to mechanical stimulation of the spinal cord. This experiment also was exceptional in that the muscles failed to relax during the ¾ sec. intermissions in stimulation. The results from this experiment were discarded.

The technique of the final myographic investigation has already been described [Eccles, 1941]. In two experiments the tenotomized tendon of soleus had lost attachment to the muscle fibres to such an extent that it tore away during a maximum tetanus. There were indications that this would have been a usual occurrence with all tenotomized muscles had the experiment been prolonged for a few more days. The muscle fibres appeared to be developing attachments to the new tendon which was being laid down around the old. Some care is necessary in order to record the maximum contraction tensions of the muscles. In all cases the values given are at the optimum frequency and initial tension, and the single and double twitches are recorded after the full development of 'treppe' for stimuli every 5 sec. 'Treppe' is particularly large with gastrocnemius.

Both the moist and dry muscle weights were determined as before, but no further reference will be made to the dry weights since the results were similar with dry or wet weights.

Results

Disuse atrophy

A. General description

The atrophic changes observed in the disused muscle at the end of 3 weeks closely resembled those already described both qualitatively and quantitatively [Eccles, 1941]. Usually the muscles were flaccid and completely quiescent, but in some fibrillation was observed, although it was rarely as intense as in muscle deprived of its motor innervation. Tower, Bodian & Howe [1941] similarly observed fibrillation in the hind limb muscles of a monkey after isolation of the lumbar region of the spinal cord, and showed that it was a true denervation fibrillation, both the cord and the sciatic nerve having degenerated. Fibrillation was not observed after a similar operation on another monkey in which the spinal cord and sciatic nerve remained normal. In our experiments the fibrillation seemed likewise to be produced secondarily to degeneration of the cord and nerve, and not to result from a pure disuse atrophy [Eccles, 1941]. Thus, in one experiment, stimulation of the motor nerves produced little or no muscular contraction on either side, and fibrillation was intense in all muscles. In four experiments intense fibrillation was observed in some muscles and was associated with low values for the contraction tension per unit muscle weight. In six further experiments slight fibrillation was noticed in one or two muscles but the contraction tension per unit muscle weight was not abnormally low. Finally, in seventeen experiments no fibrillation could be detected in any muscle. In only one experiment was it likely that mechanical damage to the cord or ventral roots occurred during the initial operation. In the other experiments showing results attributable to nerve degeneration it is probable that the ventral horn cells had degenerated on account of the unavoidable operative interference with the blood supply to the cord. All experiments were rejected in which the intensity of the fibrillation, together with the loss of contractile power, showed that a significant part of the nerve supply had degenerated.

Since the present results on disuse atrophy agree closely with those previously published [Eccles, 1941], both series have been assembled together in the averaged values shown in Table 1. Column 2 gives the average ratios obtaining for normal muscles, and in all the other columns the average values are expressed as percentages of these normal standard values. The scattering of the results approximates to the normal distribution of a population. The standard deviations are given in brackets in the Table and show that there is usually a considerable scatter in the results. This seems inevitable in experiments of this character on cats varying widely in type, weight and age. However, the chief results stand out clearly above the scatter of the individual

TABLE 1. Mean values of all experiments are shown together with standard deviations (in brackets). Values of columns 3–8 are expressed as percentages of the normal standard shown in column 2. In each column the number of experiments with a muscle is shown by the number preceding the initial letter of the muscle, e.g. 13S of column 4 means that the values of column 4 in Tables 1 *a*, *b* and *c* were derived from thirteen experiments on soleus

			Daily intermittent tetanic stimulation				
	Normal standard 12S, 12G, 12E, 12T	Disuse atrophy alone: 13S, 13G, 14E, 14T	Tenotomized muscle: 13S 16G, 5E, 5T	Maximum physiological shortening: 27S, 26G, 9E, 9T	Maximum physiological lengthening: 24E, 24T	Shortening during stimulation: 7S, 6G, 6E, 6T	Lengthening during stimulation: 5E, 5T
1	2	3	4	5	6	7	8
(*a*) $\dfrac{\text{Muscle weight in g.}}{\text{Total body weight in kg.}}$							
Soleus	1·00 (0·15)	50 (7)	58 (13)	59 (8)		75 (15)	
Gastrocnemius	3·05 (0·27)	59 (8)	62 (9)	69 (10)		83 (10)	
E.D.L.	1·06 (0·12)	64 (11)	90 (11)	96 (17)	100 (14)	107 (28)	105 (17)
T.A.	1·97 (0·18)	56 (14)	82 (14)	89 (19)	102 (16)	92 (17)	97 (11)
(*b*) $\dfrac{\text{Maximum tetanic contraction in kg.}}{\text{Muscle weight in g.}}$							
Soleus	0·65 (0·05)	71 (9)	75 (20)	72 (22)		87 (13)	
Gastrocnemius	1·27 (0·13)	79 (12)	78 (19)	87 (12)		95 (6)	
E.D.L.	0·67* (0·10)	60 (8)	92 (16)	91 (25)	81 (14)	88 (13)	82 (20)
T.A.	0·58 (0·04)	58 (12)	90 (17)	83 (13)	71 (14)	77 (16)	77 (20)
(*c*) $\dfrac{\text{Maximum tetanic contraction}}{\text{Maximum twitch contraction}}$							
Soleus	4·0 (0·8)	72 (17)	79 (9)	72 (18)		80 (14)	
Gastrocnemius	4·2 (0·7)	70 (14)	70 (10)	70 (12)		75 (7)	
E.D.L.	4·2 (0·9)	49 (7)	79 (11)	59 (13)	55 (10)	60 (8)	56 (6)
T.A.	3·5 (0·8)	51 (8)	73 (17)	58 (9)	58 (12)	63 (8)	63 (6)

* This value is low because, during the immediately preceding myographic examination of the T.A., the isolated E.D.L. contracted against no load. If this extreme shortening is prevented, the E.D.L. gives a much higher value (mean 0·98).

experiments. In addition, it has been possible to subject some of the more important series of experiments to statistical analysis. The ratio

$$\frac{\text{double twitch contraction}}{\text{single twitch contraction}}$$

has not been considered in the present paper as its relative changes closely resemble those of the $\frac{\text{tetanic contraction}}{\text{single twitch contraction}}$ ratio [Tables 4 and 5, Eccles, 1941].

Table 1, column 3 shows that, after 3 weeks' disuse, the soleus atrophied rather more than the other muscles. At the same time the contraction tension per unit weight and the tetanus-twitch ratio were low in all muscles, particularly E.D.L. and T.A. The tetanic response also differed from normal, especially with the latter two muscles, in that it was very poorly maintained during the stimulation, often falling to one-half in 1 to 2 sec. In all these respects the combined series agrees closely with the previous series. The values of column 3 may therefore be regarded as establishing satisfactory standards for the muscle changes occurring after 3 weeks of total disuse.

B. The effect of stimulation on disuse atrophy

In the previous series of experiments the much stronger ankle extensors caused the ankle to assume a position of extreme extension during the daily tetanic stimulations, and this position was maintained during the ½ sec. intermissions in the stimulation. Thus, throughout the stimulation, the extensors remained in a position of maximum physiological shortening and the flexors in a position of maximum physiological lengthening. Similar conditions prevailed in many experiments of the present series—usually in the 'control' limb—in eighteen for the extensor muscles and in sixteen for the flexor muscles. Since the results closely agreed with those of the previous series, the averages of the whole series were calculated and are shown in Table 1, column 5 for the extensors (soleus and gastrocnemius), and in column 6 for the flexors (E.D.L. and T.A.).

No systematic attempt has been made to confirm the observations of the previous paper that daily stimulations as short as 10 sec. were as effective as stimulations lasting up to 2 hr. In the present experiments daily stimulations of 4 min. proved to be slightly more effective on the average than those of 30 sec., but the results were not statistically significant.

In so far as the effect of stimulation on weight is concerned, the flexors, with full maintenance of normal weight (Table 1 *a*, col. 6), are in striking contrast to the extensors (Table 1 *a*, col. 5) where less than 25% of the weight loss is prevented. With the other ratios (Table 1 *b*, *c*), the flexors show, on the whole, a greater improvement, but the effect is much less striking than with weight.

1. *Effect of the length of the muscle on the response to daily stimulation*

The following experiments have been performed: (i) Cutting the tendo Achilles and the tendons of the other ankle extensors. Stimulation of the extensors then causes them to shorten still further than the maximum physiological shortening. At the same time the ankle was held fully flexed during stimulation in order that the flexors might assume full physiological shortening during their daily contractions. (ii) Tenotomy of the flexor tendons so that the flexor muscles might assume a still more shortened state during the daily contractions.

Columns 4, 5 and 6 of Table 1 show the response of the flexor muscles to daily stimulation under the three different conditions of length. In general the intermediate length of the muscle (maximum physiological shortening) gives values in between the shortest (tenotomized muscle) and the longest (maximum physiological lengthening).

In some experiments the flexor muscles of one limb were stimulated at a length different from that of the same muscles in the other limb. Corresponding muscles of opposite limbs so closely resemble each other that they may be regarded as initially identical; hence it has been possible to calculate the statistical significance of the differences observed between the various means [Fisher, 1934, pp. 117–20]. Table 2 shows the five experiments in which the left and right flexor muscles were stimulated in the tenotomized and fully lengthened conditions respectively. Calculation shows that there is less chance than 1 in 100 that a stimulated tenotomized muscle (either E.D.L. or T.A.) will be larger than if it were stimulated in the fully lengthened condition. The difference between columns 4 and 6 in Table 1 *a* is thus highly significant. The results of the same five experiments were more variable for the values shown in Table 1 *b*, *c*. In every respect E.D.L. and T.A. gave closely similar results, hence it seems justifiable to pool them for statistical calculation. The five experiments then show that there is about 1 chance in 15 that the contraction tension : weight ratio would be smaller for the tenotomized muscle than for the fully lengthened muscle, and about 1 chance in 30 that the tetanus : twitch

TABLE 2. The (muscle wt. in g.)/(body wt. in kg.) ratio is shown for each muscle, the value for the left (tenotomized) being shown above the right (normal, i.e. not tenotomized and showing maximum physiological lengthening). Below these values is shown the percentage change in weight for each pair as a result of the tenotomy

Exp. no.	1	2	3	23	24	Mean percentage change
E.D.L.: Left (tenotomized)	0·96	1·17	0·84	0·92	0·90	
Right (normal)	1·23	1·48	1·005	1·10	1·00	
Percentage change	−22	−21	−16	−16	−10	−17
T.A.: Left (tenotomized)	1·56	2·04	1·85	1·32	1·30	
Right (normal)	2·47	2·67	2·41	1·98	1·97	
Percentage change	−27	−20	−23	−38	−39	−31

ratio would be smaller. Thus, on the whole, it would appear that in Table 1 *b*, *c* the larger values for flexor muscles of column 4 when compared with those of column 6 are significant.

In six experiments the gastrocnemius was stimulated in the tenotomized condition on one side and in maximum physiological shortening on the other. The results, calculated statistically as above, were not significant, showing about 1 chance in 10 that the tenotomized muscle would be greater in weight, and 1 in 15 that the ratio of maximum tetanic contraction to muscle weight would be larger. Possibly the lower mean values for the tenotomized muscles achieve significance when combined with the much larger number of experiments used in calculating the gastrocnemius averages shown in Table 1 *a*, *b*. The ratio of tetanus to twitch showed no significant change in the six experiments, a result in agreement with the similar mean values in columns 4 and 5 of Table 1*c*. Since tenotomy of the soleus was usually performed on both sides, a similar statistical comparison could be made only in three experiments and was not significant for any values. However, in three experiments with tenotomy of all the ankle extensors except soleus, the contracting soleus was held in a moderately lengthened condition by the simultaneously contracting flexors. Its atrophy was significantly less than in the control limb, where it contracted in the fully shortened condition, the means being 82% as against 57% of the normal value.

The effect of length on the disused and artificially stimulated muscle may be summed up as follows.

(i) The longer the flexor muscle, and possibly also the gastrocnemius and soleus, the better is the maintenance of its weight (columns 4, 5, 6, Table 1*a*).

(ii) The longer the flexor muscle, the poorer is the maintenance of the contraction tension per unit weight. With the gastrocnemius possibly the reverse condition may occur (columns 4, 5, 6, Table 1*b*).

(iii) The longer the flexor muscle, the poorer is the maintenance of the tetanus : twitch ratio (columns 4, 5, 6, Table 1*c*).

(iv) The much greater effectiveness of stimulation in maintaining the weights of the flexor muscles, as compared with extensors, is largely dependent on some factor other than the relative lengths at which the stimulated flexor and extensor muscles are maintained (columns 4, 5, Table 1*a*).

(v) The differences between the effectiveness of stimulation with the flexor and the extensor muscles also seem significant for the contraction tension per unit weight of stimulated tenotomized muscle (column 4, Table 1*b*). This further suggests that some factor other than length distinguished the stimulated flexor from the stimulated extensor muscles, for both types of muscles are fully shortened when tenotomized.

2. *Effect of movements of a muscle during the daily stimulation*

(*a*) *Shortening during stimulation*

In these experiments the attempt was made to allow the muscles to shorten during their contraction in response to the daily stimulation. Since the maximal stimulation through the skin was necessarily applied to the whole sciatic nerve, tenotomy of all ankle extensors was essential in order to allow the weak flexors to shorten during stimulation. During the daily stimulations the limb above the ankle was fixed in a frame and the ankle was subjected to a constant mechanical pull tending to keep it fully extended. This was done by attaching a band around the toes to a pulley wheel whose axle was torsed by a heavy weight (1 kg.) working at a mechanical disadvantage of 1-5. The flexor muscles thus contracted isotonically, and during each ¼ sec. intermission they were rapidly and fully lengthened because the mechanical pull was exerted with a relatively small inertial factor. The angular movement of the ankle varied from 90 to 50° in the six experiments, and always had declined to about one-half by the end of the daily stimulation. Shortening of the extensor muscles during stimulation was brought about by a similar mechanical device working in the opposite direction, i.e. flexing the ankle during the intermission. It was not necessary to tenotomize the weak ankle flexors, but this was done in two experiments. The angular movements varied from about 90 to 50° in different experiments, but the decline by the end of the daily stimulation was much less than with the flexor muscles.

Table 1*a*, column 7, shows that with shortening during stimulation the weights of the extensor muscles were better maintained than under any other conditions, though still much below normal values. With the flexor muscles the weights were probably maintained at full normal value. With the extensor muscles the contraction tension : weight ratios and the tetanus : twitch ratios were also higher than under any other conditions (Table 1 *b*, *c*). On the other hand the flexor muscles gave lower values, which were considerably below those for tenotomized muscles.

It has been possible to calculate the statistical significance of these averaged results, since, during the daily stimulation, the opposite limb was always held so that the muscles in question contracted under fully shortened conditions (comparison of columns 7 and 5 of Table 1). This was possible with the flexor muscles because the extensor muscles of both limbs had been tenotomized.

(i) *Ratio of muscle weight to body weight.* With soleus the shortening during stimulation gives an increase which is highly significant; less than 1 in 100 would show a decrease. With the other muscles the corresponding chances of a decrease are 1 in 10, 1 in 14 and 1 in 18. The last two values (for E.D.L. and T.A. respectively) become highly significant (much less than 1 in 100) if they are pooled. In view of the similar average behaviour of all four muscles, it seems safe to conclude that, when the muscle is allowed to shorten during the daily stimulations, the weight is better maintained than if it is fixed in the fully shortened position.

(ii) *Ratio of contraction tension to muscle weight.* With soleus the increase is possibly significant. About 1 in 18 would show a decrease, but in the other muscles the results are not significant for either increase or decrease.

(iii) *Ratio of tetanus to twitch.* The increases are not significant with any muscle, and do not even achieve significance when the E.D.L. and T.A. results are pooled. In all muscles there is a chance varying from 1 in 3 to 1 in 8 that the ratio will be lower when shortening is allowed during stimulation.

(*b*) *Lengthening during stimulation*

It has only been possible to investigate this with the flexor muscles. When the limb with all tendons intact is flexed by the mechanical device during the intermission in stimulation, the contraction of the stronger extensors easily extends the ankle and so lengthens the flexors during their contraction period. Column 8, Table 1, shows that muscles stimulated under these conditions do not differ appreciably from those stimulated at a fixed, fully lengthened, condition.

C. *Effect of passive movements on disuse atrophy*

In two experiments no stimulation was applied to either limb during the 3 weeks of disuse atrophy. Both hind limbs were kept immobilized in plaster, which was removed for the daily treatment with passive movements. The ankle of one side was alternately flexed and extended every second for 4 min. The plaster was also removed from the other side for a similar period, but no passive movements were applied.

These experiments showed that passive movement had little or no effect on the course of disuse atrophy. For example, the passively exercised muscles varied in size from 16% larger to 10% smaller than the control resting side, with an average effect for the eight muscles of 2% increase. Further experiments would be necessary to determine whether passive movements have a small beneficial effect.

Tenotomy

In four experiments both the ankle extensors and flexors were tenotomized in one limb only, and the animal remained otherwise normal for 3 weeks, there being no cord operation. On dissection at the end of this period the muscles were found to be retracted and to be developing new tendons around and between the old stumps. With the ankle in a position between flexion and extension the gaps in the tendons averaged 1 cm. for tendo Achilles, 2·0 cm. for E.D.L., and 2·5 cm. for T.A. The muscles of both sides were myographically investigated in the usual way. The optimum muscle length for contraction was only gradually obtained by lengthening the muscle after each series of twitches and double twitches. The initial tension declined during each such series, so it was possible further to lengthen the muscle. The maximum contraction tension was thus recorded at a muscle length considerably longer than the resting length of the tenotomized muscle.

TABLE 3. The mean ratios for the tenotomized muscles are expressed as percentages of the mean ratios for the opposite normal muscles. Standard deviations are shown in brackets

	Muscle weight / Body weight	Maximum tetanic contraction / Muscle weight	Maximum tetanic contraction / Maximum twitch contraction
1	2	3	4
Soleus	49 (5)	61 (4)	64 (8)
Gastrocnemius	79 (5)	60 (10)	76 (9)
E.D.L.	80 (6)	89 (14)	89 (20)
T.A.	81 (6)	85 (12)	79 (9)

It will be seen from Table 3 that in respect of each measurement the values obtained are lower for tenotomized muscles than for the corresponding normal muscles of the control limb. Statistically this lowering is highly significant for the muscle weights in column 2, there being in every case much less than 1 chance in 100 of tenotomy causing an increased weight. Similarly highly significant decreases are shown for soleus and gastrocnemius in columns 3 and 4. Finally, if the E.D.L. and T.A. results are pooled, the diminutions of contraction tension per unit weight and of the tetanus : twitch ratio also become highly significant, there being less than 1 chance in 50 that tenotomy would cause an increase.

Comparison of Table 3 with Table 1, column 3, shows that tenotomy and disuse atrophy cause a similar weight loss of about 50% in soleus, but in the other muscles the weight loss resulting from tenotomy is only about 20% as compared with the 40% loss with disuse atrophy. With soleus and gastrocnemius, tenotomy causes a loss of 40% in contraction tension per unit weight, which is even higher than in disuse atrophy (20–30%).

DISCUSSION

Disuse atrophy. With the exception of the weights of the flexors, the normal condition of the muscles was not maintained by any type of daily stimulation. Two hours of stimulation were not found to be more effective than a few seconds [Eccles, 1941]. As has been shown above, the principal variations in the mechanical conditions of contraction have been tried without success. In one respect, however, the experimental conditions depart from normal. Experimentally the muscles are subjected to one intense period of activity per day, while muscles normally experience less intense activity spread irregularly over most of the day. This difference would apply particularly to the postural and antigravity muscles, soleus and gastrocnemius. This may account for the fact that, on the whole and as seen in Table 1, the gastrocnemius more closely resembled the soleus, though it is a fast pale muscle of the same type as E.D.L. and T.A. [Denny-Brown, 1929].

From the practical aspect of prophylactic treatment it would seem that disuse atrophy is best counteracted by allowing muscles to shorten during their contraction. This would simply mean active movements of the limb in question. The results suggest that, where patients are confined to bed, limb muscles may be maintained in good condition by the patient exercising them for a couple of minutes each day by *strong* voluntary contractions with movements. If movements are inadvisable, e.g. in the case of some fractures and joint injuries, or where a plaster cannot be temporarily removed, then *strong* voluntary contractions without movements are also reasonably effective in maintaining the condition of the muscles. The experimental investigation has, however, failed to discover a form of artificial stimulation which will maintain muscles in a fully normal condition. In particular, the tetanus : twitch ratio, i.e. the power of the muscle to build up a tetanic contraction, remains poor under all conditions.

Recently, Solandt & Magladery [1941, 1942], and Fischer [1941] have shown that, after section of the spinal cord in the mid-thoracic region, the gastrocnemius-soleus muscles of rats show an initial period of atrophy as intense as in the denervated muscle. However, after 2 weeks there was regression of the atrophy and by eight weeks the normal weight was almost regained. Solandt & Magladery suggest that such a regression of atrophy may also have occurred in the absolutely disused muscles prepared by Tower's method. However, in the three dogs originally prepared by Tower [1937] there was gross atrophy of the disused muscles at 2, 5 and 6 months, and in one cat, after 45 days of disuse, the atrophy was on the whole more marked (soleus atrophy to 39% normal) than the average at 21 days [Eccles, 1941]. The most probable explanation of the regression in atrophy 2 weeks after sectioning the rat's cord, is the recovery of the local reflex activity of the cord after an initial period of spinal shock [Liddell, 1934]. Solandt & Magladery report occasional bursts of activity (mass reflexes), and even suggest the presence of a low level of reflex activity, but think that this does not provide an explanation of the regression in atrophy. Since 10 sec. of daily tetanization is adequate to maintain the weights of otherwise completely inactivated flexor muscles [Eccles, 1941], it would seem that the insidious onset of reflex activity, as spinal shock passes off, would suffice to account for the regression in atrophy. Fischer states that the muscles gaining in weight were spastic.

Tenotomy. Two explanations have been offered for the atrophy of tenotomized muscles. (i) That the muscle atrophies on account of a state of overactivity called a myostatic contraction, which, it is suggested, is initially reflex in character but later becomes set in contracture [Ranson & Sams, 1928; Davenport & Ranson, 1930; Tower, 1939]. The claim of Bosmin [1925] that tenotomized muscle fibrillates has not been confirmed either by Tower [1939] or in the present experiments. (ii) That the muscle atrophies on account of decreased activity, either due to diminished motor nerve discharge or diminished work done by a muscle contracting without load [Lipschütz & Audova, 1921].

There is no acceptable evidence that a tenotomized muscle is reflexly excited so as to cause over-activity. The characteristically contracted condition of tenotomized muscle was observed in three experiments of the present series in which the full cord operation for disuse atrophy was combined with tenotomy of the muscles of one hind limb. These tenotomized muscles were fully shortened by a momentary tetanus as soon as their tendons were divided, and thereafter could not have been subjected to any reflex stimulation from the isolated and de-afferented spinal cord. These tenotomized and disused muscles did not differ significantly in either weight or contraction from the disused muscles of the control limb.

The alternative explanation of the atrophy of tenotomized muscle, that it is attributable to disuse resulting from reflex inactivity, is also contra-indicated by the present experiments. The results given in column 4, Table 1*a*, show that tenotomized muscle still suffers a large atrophy when it is subjected to a daily artificial stimulation.

However, this atrophy of the tenotomized and stimulated muscle (Table 1*a*, column 4) does not significantly differ (with the possible exception of the greater atrophy of the gastrocnemius) from the tenotomized muscle of the normally innervated limb (Table 3, column 2). This suggests that the important factor in causing the atrophy of tenotomy is not under-activity or over-activity, but merely the exceptionally shortened condition of the muscle. It has already been shown that with artificial stimulation the weight of such a shortened muscle remains significantly below the weight of an intact muscle either fully lengthened or allowed to shorten during stimulation (Table 1*a*, columns 4, 6, 7). Thus, even though a tenotomized muscle may be subjected to a normal degree of activity by discharges from the nervous system, the extremely shortened condition of the muscle fibres will still result in the occurrence of atrophy.

The many previous investigations on the atrophy caused by tenotomy showed a weight loss of the same order as that recorded in Table 3. The tetanic contraction tension per unit weight has apparently been measured only once previously [Fischer, 1941]. This investigator found that five gastrocnemius-soleus muscles of the rat showed no appreciable loss in tetanic contraction tension per unit weight at various periods (9, 10, 23, 28, 32 days) after tenotomy, though at the longest periods they had atrophied to one-half. Correspondingly there was no significant loss in birefringence. He inferred that the submicroscopic crystalline structure of the muscle is unimpaired and contrasts this with loss both of contractile power and birefringence in denervated muscle. On the other hand, Davenport & Ranson [1930] have shown that tenotomized muscle presents a grossly abnormal histological picture, mottled staining, wavy fibres, longitudinal striation and blurring of cross-striations. Similar histological changes have also been observed by Nageotte [1937] after prolonged tetanization of isolated muscles. Moreover, Ramsey & Street [1940] have shown that isolated muscle fibres suffer severe and irreversible damage (the δ state) when stimulated and allowed to shorten below 65% of their resting length. Their description of the microscopical appearance resembles that of Davenport & Ranson and of Nageotte, and the contractile power of the muscle was gravely impaired. Thus it is possible that Fischer's experiments differed from the present series in that his muscles did not shorten to the extent of producing this irreversible damage to the individual fibres. A further possibility is that the difference in the method of stimulation may be significant. In the present experiments the muscle was always indirectly stimulated through the nerve; in Fischer's experiments the muscle was *directly* stimulated with currents of optimal frequency and duration.

The experimental results show that the atrophy of tenotomized muscles is not much affected by either absolute rest or graded exercise. Since the abnormally shortened condition of the muscle fibres seems to be the important factor in causing atrophy, the only effective treatment is immediate suture of the divided tendons so that the muscle fibres are restored to their normal length.

SUMMARY

1. In thirty cats a further study has been made of the disuse atrophy which occurs in innervated muscles of the hind limb kept completely inactive for several weeks as the result of section of the cord in the upper lumbar region and of all the dorsal roots below this level. As previously reported, the ankle flexors and extensors atrophied to about 60% of normal in three weeks, and both the tetanic contraction tension per unit weight and the tetanus to twitch ratio fell to low values. Further, daily doses of artificial indirect stimulation largely prevented the atrophy of flexor muscles, but were found to be much less effective with extensor muscles.

2. It has now been shown that the shorter the muscle during stimulation, the less effective is daily stimulation in preventing atrophy. Thus stimulation is least effective in tenotomized muscle.

3. Muscles pulling against an isotonic load, and so allowed to shorten and lengthen during the daily stimulations, atrophy less than if stimulated in the fixed condition of maximum physiological shortening. Under such conditions the contraction response of extensor muscles is also maintained more nearly normal.

4. Even when similar mechanical conditions prevail during the daily stimulations, the extensor muscles atrophy much more than the flexors. In fact, atrophy of the flexors can be completely prevented provided they are not fully shortened during the daily stimulations. On the other hand, the

extensors tend to be less abnormal than the flexors in so far as the contraction response is concerned.

5. The difference between the flexor and extensor reactions, and the failure of daily stimulation to prevent extensor atrophy, are discussed, and also the application of the results to treatment of disuse atrophy.

6. Tenotomy of otherwise normal muscle is shown to give rise to loss of weight and of contraction response somewhat resembling those of disused muscles.

7. The current hypotheses that the atrophy of tenotomy results from over-activity or under-activity are shown to be contra-indicated by experiments on tenotomized muscles which are either disused or artificially stimulated. It is suggested that the atrophy of tenotomized muscle results from the extremely shortened state of the muscle fibres and is largely independent of the degree of their activity.

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ABSOLUTE MUSCLE FORCE IN THE ANKLE FLEXORS OF MAN

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All investigators since Weber [1846] are agreed that absolute muscle force should be regarded as tension per unit cross-section of the muscle because, as Weber indicated, the power of a muscle depends on its cross-section and not on its length. They are not agreed, however, on the exact definition of absolute muscle force. When the muscle is exerting its effort, should its fibres be at full stretch [Reys, 1915], half-way between full stretch and full shortening [Fick, 1910], or at 'the most favourable length' [Franke, 1920]? The results differ according to the criterion accepted. In many respects the methods previously used are unsound. The present study aims at establishing results on a more reliable basis.

The greatest resistance, against which plantar flexion of the ankle acts, can be determined and then translated into the maximum tension generated in the muscles producing the movement. Previous workers have increased the resistance by adding to the weight of the body [Weber, 1846; Reys, 1915] by applying a downward force to the lower end of the thigh [Hermann, 1898], or by an upward pressure on the ball of the foot [Reys, 1915]. Since the last appears to be the most precise method it was the one chosen for the present investigation.

METHODS

Maximum muscle force. The apparatus is shown in Fig. 1. The support behind the lumbar spine and pelvis was adjusted so that the knee could just be brought to the fully extended position. At this point the heel could be raised from the plank only by a maximum effort of ankle flexion. When the heel just cleared the plank an almost completely isometric contraction of the calf muscles was taking place in the position midway between full extension and full contraction. The leg and thigh were clear of the ground throughout the test. The distance of the head of the first metatarsal bone from the hinge was one-fifth of that between the hinge and the attachment of the spring balance, so that the pressure on the ball of the foot was five times as great as the tension in the balance.

Rapid readings, made possible by use of a spring balance, are desirable in view of the quick onset of fatigue during strong muscle contractions. Reys [1915] used weights instead of a spring and he tested both legs together. His results for the pressure on the ball of the foot were only slightly higher than those of the present study, despite the fact that he was testing professional athletes.

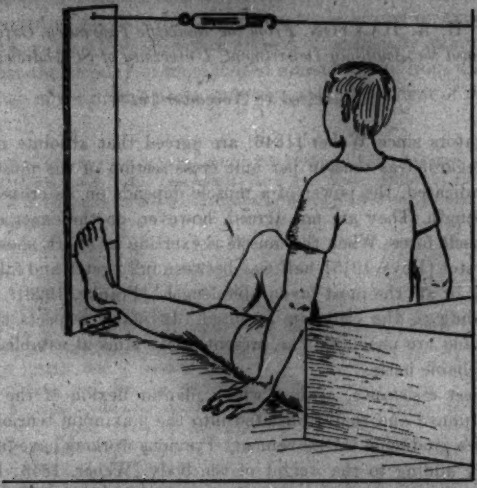

Fig. 1. Apparatus for measuring the maximum pressure on the ball of the foot against which ankle flexion can be exerted.

In order to obtain the actual tension in the calf muscles the foot must be studied as a lever. It matters not whether it is considered as a lever of the first class with the fulcrum at the ankle, as was done by the earlier workers, or as one of the second class with the fulcrum at the head of the first metatarsal [Reys, 1915; Martin, 1934], since, in equilibrium, the sum of the moments about any point is zero. The long controversy about the situation of the fulcrum does not, therefore, affect the calculation.

Five legs from anatomical subjects were cleared of all obstructing structures until free movement took place at the ankle joint with a minimum of friction. Each leg was held in clamps and spring balances were attached to the tendo calcaneus and to the foot at the level of the head of the first metatarsal bone

(Fig. 2). The readings on the two balances were taken when the foot had been pulled into slight plantar flexion by traction on the balance attached to the tendo calcaneus.

Cross-section of the ankle flexors in the cadaver. Weber [1846] estimated the area of cross-section of the calf muscles by dividing the volume by the length. It was realized by later workers that this gave only the mean anatomical cross-section, or area of section at right angles to the long axis of the muscles, and that what was required was the total physiological cross-section, or area of section of all the fibres at right angles to their long axes. The measurement of the latter, in muscles of complicated structure and with varying direction of fibre, is a difficult matter, and the methods used by Buchner [cited by Fick, 1910], Hermann [1898], Grohmann & Fick [see Fick, 1910], Reys [1915], and Franke [1920] must all be regarded as inaccurate.

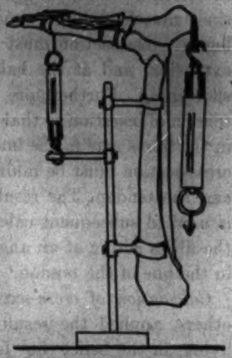

Fig. 2. Method of determining the ratio between the tension in the tendo calcaneus and the pressure on the ball of the foot.

The only way in which the physiological cross-section of a muscle can be found is to break up the muscle into bundles of parallel fibres and then to determine the total cross-section of these bundles. Examination of the gastrocnemius and soleus muscles revealed that they can be divided into a series of strips the component fibres of which are in a unipennate arrangement. In the case of the gastrocnemius two such strips, one for each head, were obtained, and in the soleus three. In each strip the proximal fibres were sectioned

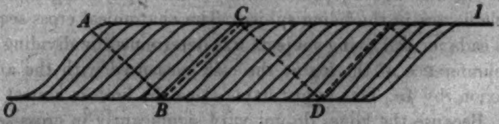

Fig. 3. Method of sectioning a unipennate muscle to obtain the physiological cross-section (see text). *OBD* is the tendon of origin and *ACI* the tendon of insertion of the muscle fibres.

at right angles to their length (*AB* in Fig. 3). At *B* a plane of cleavage *BC* was found between the last fibres cut and the first fibres still attached to the tendon of origin, and the portion *ABC* was detached. The remainder of the strip was sectioned and split alternately until all the fibres had been cut across. The total area of the cut surfaces *AB, CD,* etc., was determined by making outline tracings on celluloid of a uniform thickness, as recommended by Scammon & Scott [1927] and by Mainland [1929, 1933, 1934]. Six tracings were made from each surface in order to reduce errors due to technique. Each tracing was cut out and weighed, and the area was calculated from the weight. These cadaver muscles were in the usual fully contracted position of complete plantar flexion, and allowance had to be made for this. Since human muscle fibres contract by 57% of their extended length [Haines, 1934], and since muscle volume remains constant [Glisson, 17th century], the physiological cross-section must diminish by 57% between full shortening and full extension, and at the half-way position it is 72% of the area at complete shortening. Furthermore, the muscle fibres are inclined at an angle to the tendon of insertion so that only a resolved component of the tension developed in the fibres acts in the line of the tendon. To allow for this, the physiological cross-section must be multiplied by the cosine of the angle between the fibres and the tendon. The result, called the 'reduced physiological cross-section', is used in subsequent calculations. In effect, this manœuvre substitutes for the fibres acting at an angle to the tendon a bundle of smaller section acting in the line of the tendon.

Calculation of cross-section of the ankle flexors in life. Fick [1910], and others, applied the results obtained from cadaver muscles directly to the living, in the belief that formalin fixation produces no change in the dimensions of muscles. In the preserved legs used in the present work, however, the circumference in the region of the calf was much less than that in living subjects with a minimum of superficial fascia, so it was obvious that, in these specimens at least, the muscles were of smaller cross-section than those of the living subjects. To overcome this difficulty, the reduced physiological cross-section of the ankle flexors in the cadaver was divided by the anatomical cross-section of the cadaver leg in the region of the calf. The resulting figure was then multiplied by the anatomical cross-section of the living leg at the same level. The final figure represented the reduced physiological cross-section of the ankle flexors in the living subject. The anatomical cross-section of the leg both in cadaver and living subjects was determined by dividing the square of the circumference of the leg at the calf, measured with the ankle in full plantar flexion, by 4π. This is justifiable since the limb is almost circular at that level. Because the bones do not vary significantly in cross-section they were separated from the variable cross-section by subtraction of their sectional area.

RESULTS

The ratio of the tension in the tendo calcaneus to the resistance at the ball of the foot, determined from cadaver limbs, was $2{\cdot}67 \pm 0{\cdot}038$ (S.E. of mean of 5 observations) to 1. The constancy of this ratio allows of its application to living subjects in calculating the tension in the tendo calcaneus (Table 1).

The different cross-sections in the cadaver limbs are shown in Table 2. For the calculation of the reduced physiological cross-section the angle be-

TABLE 1. The maximum tension developed in the tendo calcaneus with the ankle half-way between full extension and flexion, obtained by multiplying the reading on the balance by 5 × 2·67

	Reading		Maximum tension	
Subject	Right leg kg.	Left leg kg.	Right leg kg.	Left leg kg.
R.G.	35	36	467·2	480·5
A.H.	37	35	493·8	467·2
M.H.	24	23	327·8	314·5
J.W.	30	30	400·5	400·5
D.K.	33	34	440·5	453·8
C.H.	37	39	493·8	520·5

TABLE 2. The relation of the reduced physiological cross-section of the ankle flexors to the anatomical cross-section of the leg in preserved limbs

Specimen	Physiol. C.S. sq.cm.	Red. phys. C.S. 'A' sq.cm.	Circumference cm.	Anat. C.S. 'B' sq.cm.	A/B
1	77·6	50·0	23·5	35·7	1·29
2	91·6	59·5	25·5	46·5	1·28
3	88·7	57·0	24·7	43·5	1·32
4	87·5	56·1	24·3	41·8	1·34

tween the muscle fibres and the tendon of insertion was found to be 10·5° for the gastrocnemius and 25° for the soleus, with the muscles in the mid-position. The ratio of the reduced physiological cross-section to the anatomical cross-section in the cadaver was a fairly constant one of 1·31 ± 0·024 (4) to 1. It seemed justifiable to assume that it holds for living subjects with a minimum of superficial fascia, and so it was used in calculating the reduced physiological cross-section in life from the anatomical cross-section (Table 3).

TABLE 3. The reduced physiological cross-section in living subjects, obtained by multiplying the anatomical cross-section by 1·31, and the absolute muscle force for the ankle flexors in the mid-position

Subject	Leg	Circumference cm.	Anat. C.S. sq.cm.	Red. phys. C.S. sq.cm.	Absolute muscle force kg./sq.cm.
R.G.	Right	35·9	97·3	127·5	3·66
	Left	35·6	95·6	125·2	3·83
A.H.	Right	35·5	95·0	124·5	3·97
	Left	35·5	95·0	124·5	3·75
M.H.	Right	30·0	66·4	87·0	3·86
	Left	30·0	66·4	87·0	3·62
J.W.	Right	32·2	77·3	101·3	3·95
	Left	32·2	77·3	101·3	3·95
D.K.	Right	34·0	86·8	113·7	3·88
	Left	34·0	86·8	113·7	3·99
C.H.	Right	35·5	95·0	124·5	3·97
	Left	35·5	95·0	124·5	4·18

Absolute muscle force. From the maximum muscle tension and the reduced physiological cross-section the absolute muscle force for the ankle flexors in the mid position was found to be 3·9 ± 0·15 (12) kg. per sq. cm. Previous estimates of the absolute muscle force in these muscles were 0·836 kg. per sq. cm. [Weber, 1846], 5·9 kg. per sq. cm. [Knorz, cited by Franke, 1920], 6·24 kg. per sq. cm. [Hermann, 1898], 5·25 kg. per sq. cm. [Reys, 1915].

DISCUSSION

In combining data from living and preserved limbs certain assumptions must be made, for example, that the ratio of the physiological cross-section of the muscles to the anatomical cross-section of the limb is the same in living and preserved limbs. It is recognized, therefore, that the error in the results may be considerable. The method described here can, however, justifiably be claimed to give a more accurate estimate of the absolute muscle force than any previously used, because it considers the alteration in cross-section with stretching or shortening and the variations in size of the calf muscles in different individuals. In addition, previous workers have considered, from measurement of distances, that the tension in the tendo calcaneus is three times the resistance at the ball of the foot; the present method gives a more direct and more accurate result.

Only the muscles which act through the tendo calcaneus are considered to be effective in producing ankle flexion. The tendons of the other muscles on the posterior aspect of the leg pass so close to the axis of the ankle joint that they act on that joint at an overwhelming mechanical disadvantage. Such is also the opinion of Reys [1915].

Since the power of a muscle decreases as the muscle shortens (von Schwann's law) and at the same time the physiological cross-section increases, it is obvious that the force per unit cross-section must decrease with shortening. Absolute muscle force, therefore, varies with the different positions of a muscle and should be defined as the series of maximum tensions produced by voluntary contraction per unit of physiological cross-section in all positions between the maximum and minimum normal lengths of the muscle. The result has here been determined for one position of the calf muscles, since changes in the ankle joint angle are difficult to measure with accuracy.

SUMMARY

1. Data obtained from living and preserved legs were used to determine (*a*) the maximum muscle force, (*b*) the physiological cross-section, and (*c*) the absolute muscle force of the ankle flexors in man.

2. When the calf muscles were half-way between full extension and full contraction the maximum muscle force developed in the tendo calcaneus of six subjects was of the order of 438 kg. The physiological cross-section averaged 112·9 sq.cm., and the absolute muscle force was 3·9 kg. per sq.cm.

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THE ACID-LABILE CO_2 IN MAMMALIAN MUSCLE AND THE *p*H OF THE MUSCLE FIBRE

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Wallace & Hastings [1942] and Wallace & Lowry [1942] have obtained relatively high values for the CO_2 content of resting mammalian muscle, which at first sight appear to be inconsistent with the theory concerning the distribution of ions in frog muscle, as presented from this laboratory [Conway & Boyle, 1939; Boyle & Conway, 1941]. With their figure of about 11 mM./kg. of total CO_2 in resting mammalian muscle, and allowing the free CO_2 to be in approximately equal concentration within and without the fibre, and the remainder of the total acid-labile CO_2 to be HCO_3^-, it follows that the ratio of HCO_3^- concentrations across the membrane would be far higher than that expected from a Donnan relation with the K concentrations. Therefore, either the mammalian muscle fibre has a very different electrolyte distribution and a different membrane permeability from that of frog muscle, or the fraction of the total CO_2 assigned by Wallace & Hastings to HCO_3^- is much too high. It was decided therefore to investigate the nature of the total available CO_2 in mammalian muscle. The solution to this problem has the further significance that it allows the *p*H value inside the muscle fibre to be determined; for whatever fraction is shown to be in truth HCO_3^- within the fibre, this, in conjunction with the free CO_2, determines the *p*H, at least within the accuracy of the pK' figure.

The following symbols are used in the calculations:

$[W]_s$ = g. water per kg. serum,
$[W]_m$ = g. water per kg. muscle,
$[HCO_3]_m$, $[HCO_3]_s$, $[HCO_3]_e$, $[HCO_3]_b$ = mM. HCO_3^- per kg. muscle, serum, extracellular fluid and whole blood respectively,
$[HCO_3]_{fw}$ = mM. HCO_3^- per kg. fibre water,
$[HCO_3]_{ew}$ = mM. HCO_3^- per kg. extracellular water,
$[HCO_3]_{sw}$ = mM. HCO_3^- per kg. serum water.

Similarly with the other ions, Na, Cl, K and H.

$[CO_2]_m$, $[CO_2]_s$, $[CO_2]_{fw}$ and $[CO_2]_{ew}$ are likewise mM. free CO_2 per kg. muscle, serum, fibre water and extracellular water,

$[CO_2, \text{total}]_m$ = the total acid-labile CO_2 per kg. muscle,

$[CO_2, \text{Ba sol.}]_m$ = the total Ba-soluble CO_2 in muscle.

The true H_2CO_3 is considered as included in the free CO_2 concentration.

METHODS

Total acid-labile CO_2 *in muscle.* The animal was anaesthetized with ether, and then a portion of the abdominal muscle quickly excised, and introduced immediately into $N/5$ CO_2-free KOH in a weighed round-bottom centrifuge tube, the cork being removed momentarily for the purpose. 25 ml. $N/5$ KOH was used as a routine in a 50 ml. tube with about 8 g. muscle. Unless the quantity of abdominal muscle was small it was cut as rapidly as possible into approximately 2 g. portions. The whole process of removal and introduction occupied no more than 10–15 sec. from the time of sectioning the muscle.

With the leg muscle of rabbit or cat, about 10 g. were quickly excised and held over the KOH tube; quantities a few mm. thick were rapidly sectioned by sharp scissors and dropped into the alkali.

The stopper was replaced, the tube weighed, and the contents well mixed. The whole was then placed in the refrigerator for an hour, being shaken several times throughout this period.

The tube was then spun for a few minutes and 0·5 ml. volumes pipetted quickly into the outer chambers of Conway units (no. 2 size), already prepared with 0·2 ml. $N/25$ $Ba(OH)_2$ containing 5 % B.D.H. universal indicator, in the central chamber, and 0·2 ml. $2N$ H_2SO_4 in the outer chamber [Conway, 1939; O'Malley, Conway & FitzGerald, 1943].

The titrations were carried out after an hour, with $N/40$ HCl from a Conway micro-burette [Conway, 1939], to a green end-point.

The determinations were made in triplicate. The large standard units (Conway unit no. 1) were also occasionally used with 2 ml. extract, 1·3 ml. $N/40$, $Ba(OH)_2$ in the central and 0·5 ml. $2N$ H_2SO_4 in the outer chamber, with subsequent titrations of 1 ml. vol. removed from the central chambers into small tubes, using $N/10$ HCl.

Blank determinations were carried out in a similar manner, a volume of CO_2-free water corresponding to the water content of the muscle being pipetted into the alkali, and carried through the whole procedure as for muscle.

Calculation of mM. CO_2/kg. *muscle.* In the calculation it is assumed that the muscle contributes its water to the total fluid volume, the membranes being under the conditions freely permeable to all electrolytes other than protein which also escapes in a certain measure. The volume corresponding to 1 g. of muscle is thus $\frac{0{\cdot}77w+25}{w}$, where 25 ml. KOH solution is used.

For the procedure described with the no. 2 units the following formula applies:

$$\text{mM. } CO_2/\text{kg. muscle} = 0{\cdot}0625x\left(3+\frac{100}{w}\right), \tag{1}$$

where x = large divisions on burette corresponding to the CO_2 absorbed, taking the blank reading as zero absorption (each large division on the burette = 0·01 c.c.).

For the procedure with the no. 1, or standard units, the formula is

$$\text{mM. } CO_2/\text{kg. muscle} = 0{\cdot}0812x\left(3+\frac{100}{w}\right). \tag{2}$$

The Ba-soluble fraction. This was determined in a similar way, but prior to the CO_2 absorptions in the micro-diffusion units 5 ml. vol. of the alkali extract were pipetted into capped 15 ml. centrifuge tubes (tapered) and 1 ml. saturated $BaCl_2$ pipetted, added and mixed. These tubes were then centrifuged at about 3000 r.p.m. for at least 90 min. For abdominal muscle of rats, very young rabbits (0·5 kg.) or guinea-pigs, there was in nearly all cases no apparent opacity left after this centrifuging and there was usually found to be extremely little on measuring with the Pulfrich Turbidimeter, the results being expressed in absolute values (using test-tubes and wedge illumination).

Total CO_2 *in blood plasma.* For this purpose the blood was collected under paraffin from a carotid cannula and centrifuged. 0·2 ml. vol. were pipetted into the outer chambers of Conway units (no. 2) with 0·2 ml. $N/10$ $Ba(OH_2)$ with 5 % B.D.H. universal indicator and 0·5 ml. $N/1$ H_2SO_4, in the outer chamber. The pipette used for the plasma was one delivering rapidly between two points. The procedure was controlled by similar deliveries of 0·2 ml. $M/50$ $KHCO_3$.

Chloride in plasma. This was carried out by a micro-diffusion procedure [Conway, 1935, 1939].

Chloride in muscle. The muscle was bubbled for an hour in 10 ml. 1·9 % Na_2SO_4 (anhydrous) per g. muscle. To 10 ml. of this extract in a 15 ml. centrifuge tube 1 ml. 10 % tungstate and 1 ml. $\frac{2}{3}N$ H_2SO_4 were added, mixed and the precipitate separated by centrifuging. 1 ml. vol. of the clear fluid were used for the analyses by the micro-diffusion procedure [Conway, 1939]. The procedure was varied by using 1·3 ml. 20 % KI instead of 1 ml. as previously described, and removing 1 ml. after 90 min. into a cell of the Spekker absorptiometer. 5 ml. water were added and mixed, and the readings controlled with $N/100$ HCl.

The initial immersion and bubbling with 1·9 % Na_2SO_4 was found advantageous for mammalian muscle, instead of the previously described method which was found useful for frog tissues, since grinding the mammalian muscle with tungstate and $\frac{2}{3}N$ H_2SO_4 gave a filtrate, from which the Cl emission in the micro-diffusion units was delayed. In the calculation it was assumed that under the conditions all the muscle water was freely available for interchanges, and that the Cl had the same concentration within as without owing to the breakdown of the membrane system. For the abdominal muscle used, with interspace value of about 25 %, the maximum error that could take place on this assumption is 5 % and the actual error would usually be less. This was considered sufficiently accurate for our purpose.

Blood in muscle. The $N/5$ KOH extracts all the blood from the muscle and probably small amounts of myohaemoglobin. The colour was compared with that of blood in $N/5$ KOH and suitably diluted.

Water in muscle. This was determined on weighed samples by heating for 12 hr. at 105° C.

RESULTS

Rate of the alkali extraction of total CO_2 *from muscle*

In the method described for determining the total acid-labile CO_2 in muscle, an hour was allowed for the alkali extraction. It will be seen from Fig. 1 (experiments on very young rabbits, 300–500 g. wt.) that the extraction is practically complete after this time, and for the investigations to be described subsequently, the shortest extraction period possible was desirable—consistent with the removal of all or nearly all the CO_2.

The total acid-labile CO_2 *in mammalian muscle*

Table 1 gives representative data for the total CO_2 for abdominal and leg muscle (as well as the Ba-soluble fraction and the opacity of the extract with $BaCl_2$ after centrifuging 90 min. or more).

For the rat abdominal muscle the mean total CO_2 was 14·8 mM./kg. (range of 13·4–15·9). For the eight young rabbits it was 11·4 with S.D. for the single observation of 0·7 (S.D. of mean = 0·3). For seven guinea-pigs it was 10·3 with S.D. of 1·2. The mean for the leg muscle of eight rabbits was 10·6 with

S.D. of 1·2 and for the leg muscle of two cats it was 10·1 and 10·8 mM./kg. Comparing these values with those of Wallace & Hastings [1942] using a different method [Danielson & Hastings, 1939], the mean value for the leg muscle of the cat (taking their 14 control series) was 11·0 with S.D. of 1·3, agreeing very well, therefore, with the above results.

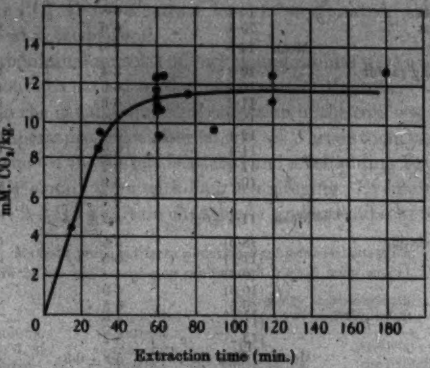

Fig. 1. Time curve of CO_2 extraction from abdominal muscles of young rabbits (about 0·5 kg.) by *N*/5 KOH. Each point in general from one rabbit; two points obtained with some rabbits.

The Ba-*soluble fraction*

The average values for the CO_2 remaining on addition of 1 vol. saturated $BaCl_2$ to 5 vol. of extract and spinning for 90 min. (occasionally much longer) at approximately 3000 r.p.m. were for the abdominal muscle of the rat, rabbit and guinea-pig, 8·0, 5·2, and 5·8 mM./kg. respectively (14·8, 11·4 and 10·3 mM./kg. being the corresponding values for the total CO_2), and for the leg muscle of rabbit and cat the value was 7·0 mM./kg. (compared with 10·6 mM./kg. for the total CO_2). Thus under the conditions of the experiment more than half the total CO_2 is not precipitated by $BaCl_2$, which is presumptive evidence that this fraction is not present in muscle as free CO_2 or HCO_3^-, or in the alkaline extract as CO_3 ion. This conclusion has been proved by two independent procedures.

(1) *Effect of addition of* $KHCO_3$ *to the muscle extracts.* To 5 ml. portions of alkaline extract of the muscle small volumes of $KHCO_3$ solution (0·1–0·2 ml.) were added and mixed before adding the saturated $BaCl_2$, corresponding volumes of CO_2-free water being added to control tubes. The results are shown in Table 2. If the $CO_3^=$ were not entirely precipitated it might be expected that higher values would be found for the analyses after centrifuging. In fact,

TABLE 1.

Animal		Total CO$_2$ mM./kg.	Total Ba-soluble CO$_2$	Turbidity of Ba extract after centrifuging, absolute units
		Abdominal muscle		
2 rats		13·4	7·4	—
2 rats		15·9	11·3	—
1 rat		—	7·9	—
3 rats		14·4	6·8	—
3 rats		15·4	6·6	0·009
	Mean	14·8	8·0	
Young rabbit		10·8	3·4	0·000
		10·7	6·6	0·000
		11·2	6·3	0·001
		12·5	6·2	0·027
		12·4	4·0	0·000
		11·4	4·2	0·010
		11·8	3·2	—
		10·7	5·9	0·000
		—	6·5	—
	Mean	11·4 ± 0·3	5·2 ± 0·4	
Guinea-pig		(8·5)	5·5	0·002
		(11·1)	6·5	0·016
		(9·4)	—	—
		(10·3)	4·9	—
		(9·5)	6·5	—
		(11·1)	6·2	—
		(12·2)	5·2	—
	Mean	10·3 ± 0·4	5·8 ± 0·3	
		Leg muscle		
Young rabbit		8·9	7·5	—
		11·2	9·1	—
		12·3	6·6	0·000
		10·7	6·4	0·000
		9·3	4·7	0·007
Grown rabbit		11·8	—	Cloudy
		9·0	7·2	—
		11·3	7·2	—
	Mean	10·6 ± 0·4	7·0 ± 0·5	
Cat		10·8	9·0	Cloudy
		10·1	7·3	"
	Mean	10·4	8·1	

The brackets for total CO$_2$ for guinea-pig abdominal muscle indicate that different animals were used for determining this quantity and for the Ba-soluble fraction. The turbidities listed give absolute values over the readings for the centrifuged control without BaCl$_2$ which usually gave results not differing from water. The ± figures after the means give the S.D. of the mean values.

TABLE 2.

		Ba-soluble CO$_2$ in extract		
Animal	Muscle	Before adding KHCO$_3$ mM./kg. muscle	After adding KHCO$_3$ mM./kg. muscle	Amount of KHCO$_3$ added as mM./kg. muscle
2 rats	Abdominal	7·4	7·0	10·5
		11·3	10·2	8·6
Rabbit	Leg	7·2	6·8	4·0
		6·6	5·0	13·0
		4·3	5·2	6·0
		4·7	3·5	5·3

slightly lower values were found. All the added HCO_3^- was precipitated as $BaCO_3$, and apparently the extra bulk of the precipitate brought down a little of the Ba-soluble fraction.

The mean value of the Ba-soluble fraction without adding $KHCO_3$ was 6·9 mM./kg., and after the addition of $KHCO_3$ (corresponding to a mean value of 7·9 mM./kg. muscle) it was 6·3 mM./kg. It then seemed possible that although all the $CO_3^=$ above a certain level is precipitated, yet some $BaCO_3$ might still be held in suspension.

To test this, abdominal muscles of very young rabbits were evacuated in the cold for some hours to get rid of the major part of the total CO_2. They were then extracted in the usual manner. Evacuation in the cold was chosen rather than at room temperature, since after 90 min. or 2 hr. at room temperature the physical consistence of the muscle is altered and after adding $BaCl_2$ the extract remains markedly cloudy even with long centrifuging. Even after evacuation for some hours at 2–3° C. a certain change of a similar kind is apparent. Table 3 shows the effect of the evacuation and of additions of $KHCO_3$ on the Ba-soluble fraction. The initial Ba-soluble fraction was 2·2 mM./kg., and after additions of $KHCO_3$ corresponding to 10·9 and 12·7 mM./kg. it was 2·8, the average of all the analyses after adding $KHCO_3$ being 2·9. This shows perhaps a slight increase after the addition but nothing comparable to the average level of 5·2 mM./kg. for the Ba-soluble fraction *in the extract of unevacuated muscle.* It may be also attributed to the fact that $BaCO_3$ is not so easily precipitated from the alkaline extract of *muscle which has been long standing.*

TABLE 3. Extract prepared from abdominal muscle—evacuated in the cold for 2½ hr.—of four young rabbits (0·3–0·5 kg. body weight)

Ba-soluble CO_2 mM./kg. muscle	$KHCO_3$ added to extract as mM./kg. muscle	Turbidity in absolute units Extract after $BaCl_2$ addition	
		Before centrifuging	After 90 min. centrifuging
2·2	0·0	0·253	0·019
2·3	1·9	0·260	0·020
3·1	3·8	0·289	0·020
3·2	5·5	0·309	0·006
2·9	7·4	0·330	0·022
3·1	9·2	0·352	0·020
2·8	10·9	0·361	0·006
2·8	12·7	0·358	0·000

(2) *Turbidity studies.* The centrifuged extracts after $BaCl_2$ addition were examined, as described, in the turbidimeter (Pulfrich), the turbidities being expressed in absolute terms. For abdominal muscle the turbidity measured was often less than 0·001, such extracts appearing quite clear to ordinary observation. When compared in the turbidimeter with centrifuged control samples containing no $BaCl_2$ there was usually no measurable difference in turbidity. The question then arose as to the turbidity produced by $KHCO_3$ additions to alkaline protein solutions, with subsequent $BaCl_2$ additions. The most suitable solution for examination seemed to be the extract of evacuated abdominal muscle in which everything was otherwise similar to the analytical conditions. Several hours' evacuation of the abdominal muscle in the cold brought the total acid-labile CO_2 to about

2 mM./kg. The muscle was then extracted in the usual manner and the $KHCO_3$ added in small measured volumes (0·1–0·2 ml.) of standard solutions to 5 ml. vol. of the extract (obtained from about 17 g. muscle in 50 ml. $N/5$ KOH). After mixing, 1 ml. saturated $BaCl_2$ was added and again rapidly mixed. It may be noted that different turbidity conditions are obtained if the $KHCO_3$ additions are made *after* the $BaCl_2$ addition. There is then present a fine flocculent precipitate, whereas with the above procedure no flocculent precipitate is produced but rather a fine cloud.

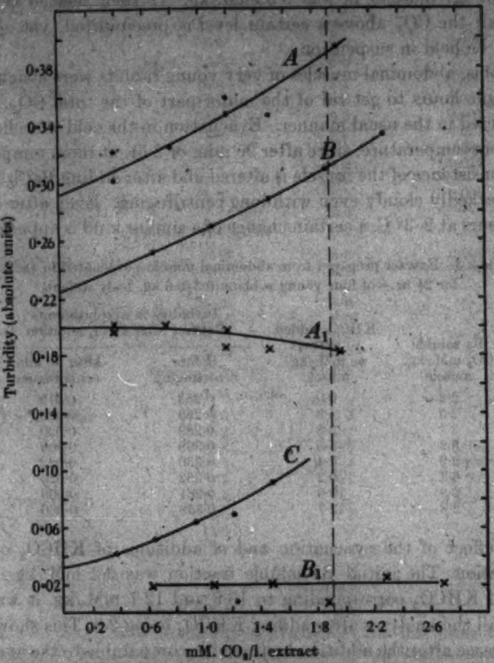

Fig. 2. Curves *A* and *B* represent turbidities of alkali extracts of evacuated abdominal muscle from young rabbits as described in text, after additions of $KHCO_3$ and then saturated $BaCl_2$, 1 vol. to 5 vol. of extract. Curve A_1 represents the *A* extracts after 5 min. centrifuging. Curve B_1 represents the *B* extracts after 90 min. centrifuging. Curve *C* is for clear human serum freed of CO_2 and rendered alkaline to simulate the muscle extracts, $KHCO_3$ added and then $BaCl_2$ as for muscle. The dilution of the serum in the alkaline fluid before $BaCl_2$ addition was about 1 in 5.

Fig. 2 shows the results obtained in two such experiments (curves *A* and *B*). The remaining small amount of acid-labile CO_2 in the muscle is here not considered to be present in the extract as $CO_3^=$, but the small amount of $CO_3^=$ in the alkali itself is added on to the $KHCO_3$ additions. The abscissae give the concentrations of carbonate present and the ordinates the turbidities in absolute values.

It will be seen that the additions cause a uniform rise in turbidity, at least up to the approximate level (dotted vertical line) of the Ba-soluble fraction of the total acid-labile CO_2 in the extracts of fresh muscle. The relation of increase in turbidity to increase in added CO_2 is nearly but not quite linear. The turbidity changes 0·10 in absolute units for a CO_2 addition corresponding to the level of the Ba-soluble fraction of fresh muscle. As already noted the turbidity of the centrifuged extracts (unevacuated muscle) after $BaCl_2$ addition is often inappreciable, averaging 0·008 for abdominal muscle, which was the muscle most examined.

Curve A_1, in Fig. 2, shows the effect on the extracts used for curve A (with $BaCl_2$ addition) of centrifuging for 5 min. The turbidity is still relatively high, but there is now a slight fall with increasing $KHCO_3$. Curve B_1 shows the effect on the B series of centrifuging for 90 min. Very little turbidity is left and there is no difference for the increasing $KHCO_3$ additions.

Curve C is for clear human serum diluted 1 in 5 with $N/5$ KOH, and which had previously been freed of CO_2 by slight acidification and exposure for an hour in micro-diffusion units. 5 ml. vol. were taken and additions of $KHCO_3$ made as above with subsequent $BaCl_2$ addition. It will be seen that the effect on the turbidity is very similar to that with extract of abdominal muscle.

The results show clearly that the comparatively large Ba-soluble fraction of the total CO_2 in the alkaline extracts cannot be present as $CO_3^=$.

The nature of the Ba-*soluble fraction*

A large fraction of the total acid-labile CO_2 in muscle is Ba-soluble in alkaline media, and this at once suggests [Henriques, 1928, 1929, 1935; Faurholt, 1924, 1925; Meldrum & Roughton, 1932, 1933; Roughton, 1935] that it may be carbamino CO_2. Now such compounds possess the characteristic property that around a pH of 7·0, when the CO_2 tension falls, they are rapidly split, yielding free CO_2.

A series of observations on the effect of exposure of strips of the abdominal muscle of guinea-pigs (numbers of which were available at the time) *in vacuo* were therefore carried out. The results of these experiments are summarized in Fig. 3 (each point being the mean of 3–6 determinations). It will be seen that both the total and Ba-soluble fraction show a rapid initial fall of 2–3 mM./kg. after which the Ba-soluble fraction falls only very slowly, and is little more than halved after full evacuation for 1 hr. at room temperature. At and after 45 min. there is no appreciable difference between the curves of the total and the Ba-soluble CO_2, so that all the free CO_2 and HCO_3^- has then disappeared from the muscle. This would seem to indicate that the greater part of the Ba-soluble fraction may not be carbamino CO_2. The mean value of the sum of the free CO_2 and HCO_3^- in the abdominal muscle of the guinea-pig is

10·3 ± 0·4 minus 5·8 ± 0·4, i.e. 4·5 ± 0·6 (see Table 1). For rabbit abdominal muscle it is 11·4 ± 0·3 minus 5·2 ± 0·3, i.e. 6·2 ± 0·4, and for the leg muscle of the rabbit it is given by 10·6 ± 0·4 minus 7·0 ± 0·5, i.e. 3·6 ± 0·6.

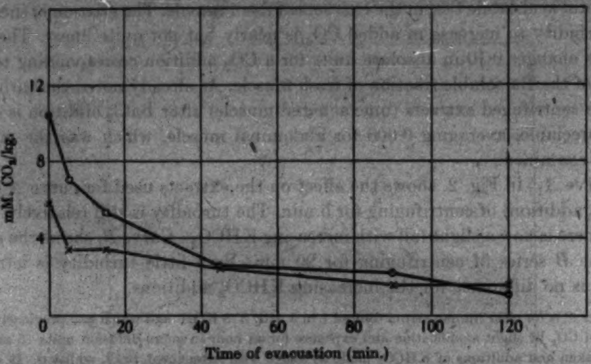

Fig. 3. Mean curves of CO_2 content of abdominal muscle of young guinea-pigs after evacuation for varying periods. Upper curve gives the total acid-labile CO_2. Lower curve gives the barium-soluble CO_2. Each point is for 3–6 analyses. Each guinea-pig was used to supply data for two time periods.

The ratio of HCO_3^- *concentration in muscle as a whole to* HCO_3^- *content of serum compared with similar ratio for chloride*

To determine this ratio for abdominal muscle we have the following data from Table 4.

The ratio $[Cl]_m/[Cl]_s$ for rabbit abdominal muscle = 26·6/99·5 = 0·267, and the ratio $[HCO_3]_m/[HCO_3]_s$ = 5·3/19·4 = 0·273. (The HCO_3^- value is obtained from Table 4 by subtracting the Ba-soluble fraction from the total acid-labile CO_2, and then a further 0·9 for the free CO_2 in solution in the total muscle water. In calculating plasma HCO_3^-, 1·0 is allowed for the free CO_2.) For the guinea-pig, assuming the same value for the free CO_2 in serum, etc.

$$[HCO_3]_m/[HCO_3]_s = 0{\cdot}186, \quad \text{and} \quad [Cl]_m/[Cl]_s = 0{\cdot}208.$$

The ratios for Cl^- and HCO_3^- are thus the same within the sampling error. If the interspace volume be calculated from the chloride analyses, assuming no chloride *inside* the fibres, there could be no intracellular bicarbonate, but such a method of interspace calculation, though giving values near to those actually found, is theoretically unsound [Conway & Boyle, 1939; Boyle & Conway, 1941]. How the true interspace volume, etc., may be calculated from chloride analyses is shown below.

TABLE 4

'Tissue'	Symbol	Value of symbol	
		Rabbit	Guinea-pig
Plasma	W_s (g./kg.)	924 (A)	—
	$[CO_2$, total$]$	20·4 (8)	—
	$[Cl]_s$	99·5 (10)	109·0 (8)
	$[K]_s$	4·9 (5)	—
	pH	7·4	—
Whole blood	W_b (g./kg.)	817 (A)	—
	$[K]_b$	45 (A)	—
	$[Cl]_b$	82 (A)	—
Muscle (abdominal)	W_m (g./kg.)	782 (5)	771 (5)
	$[CO_2$, total$]_m$	11·4 (8)	10·3 (7)
	$[CO_2$, Ba-sol.$]_m$	5·2 (9)	5·8 (6)
	$[Cl]_m$	26·6 (7)	22·7 (5)
	Blood (g./kg.)	28 (5)	29 (5)
Muscle (leg)	W_m (g./kg.)	767 (5)	767 (5)
	$[CO_2$, total$]_m$	10·6 (8)	—
	$[CO_2$, Ba-sol.$]_m$	7·0 (7)	—
	$[Cl]_m$	12·1 (6)	—
	$[K]_m$	111 (C)	—
	Blood (g./kg.)	25 (5)	24 (5)

All values as mM./kg., unless otherwise stated. The symbol $[Cl]_s$ means mM. chloride/kg. plasma and $[K]_m$ means mM. potassium/kg. muscle and similarly for the other symbols. W_s and W_m mean g. water/kg. plasma and muscle. Figures in brackets give numbers of analyses. (A) refers to Abderhalden's data [1899], and (C) to Constantino's [1911].

The pH *within the muscle fibre*

The value may be first calculated without implying a Donnan relation across the membrane.

In the Henderson-Hasselbalch equation (as given in equation (4)) $[CO_2]_{fw}$ and $[HCO_3]_{fw}$ are the concentrations of free CO_2 and HCO_3^- within the fibre (the very small value of H_2CO_3 may be neglected). A value of $pK = 6{\cdot}1$ may be assumed, as the ionic strength within the fibre will probably not differ markedly from that of blood plasma.

To calculate $[HCO_3]_{fw}$ we need to know the value of the intercellular space and this must be obtained independently of the chloride data. For the leg muscle of the rabbit we have the inulin ratio [Conway & FitzGerald, 1942] $\frac{\text{inulin/kg. muscle}}{\text{inulin/kg. plasma}} = 0{\cdot}07$. Manery & Hastings [1939] found for radioactive sodium (^{24}Na) a ratio of 0·086 with the gastrocnemius muscle of the rabbit; Hahn, Hevesey & Rebbe [1939] obtained a very similar value of 0·085.

The interchange with radioactive Na in such experiments is no doubt entirely extracellular, and it is not surprising that the inulin value should be slightly lower, as some Na may be held by fixed anions in the sarcolemma.

But, taking the sodium ratio as 0·086 for the extracellular water, this, apart from the blood in muscle, may be calculated as follows. In the calculation the mean water content of rabbit leg muscle is considered to be 767 g./kg. muscle, and the water in serum to be 920 g./kg.

If it be supposed that in 1 kg. of leg muscle there are x kg. of extracellular fluid in addition to y kg. of blood, then

$$\begin{aligned}[\mathrm{Na}]\ \text{external to the fibres} &= 0{\cdot}99x\,[\mathrm{Na}]_{ew} + y\,[\mathrm{Na}]_b \\ &= 0{\cdot}99x\,[\mathrm{Na}]_{ew} + 0{\cdot}5y\,[\mathrm{Na}]_s. \end{aligned} \qquad (3)$$

Here it is taken, in agreement with Wallace & Hastings [1942], that 1 kg. of extracellular fluid contains 0·99 kg. water, and that the sodium per kg. whole blood—$[\mathrm{Na}]_b$—is approximately half that per kg. serum or 0·5 $[\mathrm{Na}]_s$.

Now $[\mathrm{Na}]_{ew}$ may be taken as $[\mathrm{Na}]_{sw} \times (0{\cdot}95/0{\cdot}92)$, where 0·95 is the Donnan ratio and 0·92 is the water content of the serum. Using these values as well as 0·025 found by us for the blood in the excised leg muscle of the rabbit, and the figure 0·086 above, x is found from equation (3) to be 0·075.

The extracellular fluid, apart from blood, is therefore 0·075 kg./kg. muscle. The water content of this is $0{\cdot}99 \times 0{\cdot}075 = 0{\cdot}074$, and the water content of 0·025 kg. blood is $0{\cdot}025 \times 0{\cdot}77 = 0{\cdot}019$. The total extracellular water is therefore $0{\cdot}074 + 0{\cdot}019 = 0{\cdot}093$; and the intracellular water

$$0{\cdot}767 - 0{\cdot}093 = 0{\cdot}674.$$

To determine the HCO_3^- content of the intracellular water it is necessary to assess the HCO_3^- and free CO_2 in the spaces outside the fibres and the free CO_2 within the fibres. Now

$$[\mathrm{HCO_3}]_{sw}\ (\text{mM. } \mathrm{HCO_3^-}/\text{kg. of the serum water}) = 21{\cdot}0,$$

and therefore $[\mathrm{HCO_3}]_{ew}$ (mM. HCO_3^-/kg. of extracellular water) $= 21{\cdot}0 \times 1{\cdot}05 = 22{\cdot}1$. Also, $[\mathrm{CO_2}]_{sw} = 1{\cdot}1$, and since the solubility coefficient of CO_2 is 0·553 ml./g. serum water, 0·540 ml./g. extracellular water and 0·592 ml./g. intracellular water [as given by Wallace & Hastings, 1942] then $[\mathrm{CO_2}]_{ew}$ (mM. free CO_2/kg. extracellular water) $= 1{\cdot}08$, and $[\mathrm{CO_2}]_{fw}$ (mM. free CO_2/kg.) $= 1{\cdot}18$. Since the total CO_2 content of the serum is 20·4 mM./kg. (Table 4), that of whole blood may be taken as approximately 17 mM./kg. From the value of the total CO_2 content of muscle apart from the Ba-soluble fraction (that is, from $10{\cdot}6 - 7{\cdot}0 = 3{\cdot}6$ mM./kg.) we must subtract then the following to obtain the HCO_3^- content of the fibre water:

$0{\cdot}075 \times 1{\cdot}08$ ... free CO_2 in extracellular fluid other than blood,
$0{\cdot}075 \times 22{\cdot}1$ ... HCO_3^- in extracellular fluid other than blood,
$0{\cdot}025 \times 17$ ... total CO_2 in the blood in muscle,
$0{\cdot}674 \times 1{\cdot}18$ ... free CO_2 in the fibre water (or extracellular fluid)
$= 2{\cdot}96$,

so that $3{\cdot}6-2{\cdot}96=0{\cdot}6$ mM. HCO_3^- is left dissolved in 0·674 kg. water, or $[HCO_3]_{fw}=0{\cdot}6/0{\cdot}674=0{\cdot}9$. From the Henderson-Hasselbalch equation

$$pH=pK'-\log\frac{[CO_2]_{fw}}{[HCO_3]_{fw}}, \tag{4}$$

the mean value of the intracellular $pH=6{\cdot}1-\log 1{\cdot}2/0{\cdot}9=6{\cdot}0$. No great exactness can be claimed for this figure, owing to the relative magnitude of the total interspace CO_2, and of the Ba-soluble fraction inside the muscle fibre.

Calculation of the pH *within the muscle fibre from the application of the Donnan principle and assuming a membrane permeable to* K, H, Cl *and* HCO_3 *ions*

From the Donnan relation, the ratio of the potassium activities across the membrane should be the same as the ratio of the hydrogen activities, and inversely as the chloride and bicarbonate activities. Since we are dealing with univalent electrolytes, we may without much error substitute the ratio of the concentrations. Also, to simplify the presentation, the concentration of the inorganic electrolytes in the extracellular fluid may be taken to be the same as in the plasma water, since any small differences from these values would contribute no appreciable extra accuracy to the calculations, besides being in themselves of rather uncertain value. From the Donnan relation

$$\frac{[K]_{fw}}{[K]_{sw}}=\frac{[H]_{fw}}{[H]_{sw}}. \tag{5}$$

Also,

$$[K]_{fw}=\frac{[K]_m-s\times[K]_{sw}-0{\cdot}025[K]_b}{0{\cdot}767-s-0{\cdot}025\times0{\cdot}77}, \tag{6}$$

where $[K]_{fw}$, $[K]_m$, $[K]_{sw}$, $[K]_b$ are the mM. K/kg. of fibre water, of muscle, of serum water and of whole blood respectively; 's' is the volume of interspace water other than blood, which latter is 0·025 kg./kg. muscle (0·77 times this value, giving the water content) and 0·767 is the amount of water per kg. muscle from Table 4. Therefore, from the data of Table 4

$$[K]_{fw}=\frac{111-s\times5{\cdot}3-0{\cdot}025\times45}{0{\cdot}767-s-0{\cdot}019}$$

$$=\frac{110-5{\cdot}3s}{0{\cdot}748-s}, \tag{7}$$

so that (from equation (5))

$$[H]_{fw}=\frac{110-5{\cdot}3s}{0{\cdot}748-s}\times\frac{10^{-7{\cdot}4}}{5{\cdot}3}. \tag{8}$$

There remains then to calculate 's', the value of the extracellular water, apart from blood. It may be determined by using the following relation:

$$[K]_{fw}\times[Cl]_{fw}=[K]_{sw}\times[Cl]_{sw}, \tag{9}$$

when the value of $[K]_{fw}$ is inserted from equation (7), and $[Cl]_{fw}$ (as in equation (11), similar to $[K]_{fw}$) is determined as follows:

$$[Cl]_{fw} = \frac{[Cl]_{m} - s \times [Cl]_{pw} - 0{\cdot}025\,[Cl]_{b}}{0{\cdot}767 - s - 0{\cdot}025 \times 0{\cdot}77}$$

$$= \frac{12{\cdot}2 - s \times 108 - 0{\cdot}025 \times 82}{0{\cdot}748 - s}$$

$$= \frac{10{\cdot}1 - 108s}{0{\cdot}748 - s}, \tag{10}$$

whence, from equation (9),

$$\frac{110 - 5{\cdot}3s}{0{\cdot}748 - s} \times \frac{10{\cdot}1 - 108s}{0{\cdot}738 - s} = 5{\cdot}3 \times 108, \tag{11}$$

from which $s = 0{\cdot}071$,

which is very similar to the 0·075 value obtained above from direct analyses.

Inserting this value for 's' in equation (8)

$$[H]_{fw} = 10^{-5{\cdot}9},$$

and the pH value = 5·9, or, if we consider the pH value as the negative logarithm of the H ion activity (to correspond to equation 5 above) the value is slightly raised and becomes 6·0.

Here again no great exactness can be claimed for the result, but it is probably within ±0·1 pH, and agrees well with the previous calculation, based directly on the bicarbonate system.

Similar calculations, applied to the data for the abdominal muscle of the rabbit, give a pH within the fibres of 6·0, which is similar to that of the leg muscle.

DISCUSSION

Ba-*soluble* CO_2 *in muscle*

The total CO_2 liberated and escaping after acidifying muscle may be termed acid-labile CO_2; as shown here, only the smaller part of this total in mammalian muscle is in the ionized HCO_3 or in the free CO_2 form, the greater part being Ba-soluble in alkaline media.

The proofs of the reality of the existence of a Ba-soluble fraction of such magnitude, and that $BaCO_3$ is not merely suspended or protected from precipitation by the proteins, have consisted in the quantitative precipitation of small amounts of CO_2 added as $KHCO_3$ to the alkaline extract before the addition of the $BaCl_2$, as well as by turbidity studies of the centrifuged samples, and turbidity effects produced by HCO_3^- additions to evacuated muscle extracts. The proof obtained from the turbidity study alone would appear conclusive for the abdominal muscles of guinea-pigs, very young rabbits and rats. The alkali extracts of leg muscles of fully grown rabbits and cats, when treated with $BaCl_2$, show some turbidity after centrifuging, though this is no reason for supposing that such turbidity is caused by $BaCO_3$, since as with abdominal muscle this is quantitatively precipitated when small volumes of $KHCO_3$ solutions are added before the $BaCl_2$. Evidence has been presented in the paper that the greater part of this Ba-soluble fraction may be in some form other than carbamino CO_2.

The pH *inside the muscle fibre*

It will be seen that the nature of the acid-labile CO_2 in mammalian muscle as determined in these experiments altogether invalidates the pH calculation made by Wallace & Hastings, their calculated value of 6·93 ± 0·12 being nearly one whole unit of pH too high. The true value appears to be approximately 6·0, and is thus practically the same as that calculated for frog muscle, i.e. 5·9 [Boyle & Conway, 1941].

The calculations of the H-ion concentration from the study of the bicarbonate system are in agreement with a Donnan relation across the membrane for K^+, H^+, Cl^- and HCO_3^- and such a relation for K^+ and Cl^- has been demonstrated for very wide changes of these ions in frog muscle [Boyle & Conway, 1941]. Supporting evidence for mammalian muscle is given by Wilde [1943] and Darrow [1944]. It is also of interest to note that a value of 6·0 was found by Vlès [as quoted by Rous, 1925] for frozen and ground mouse tissues by various physico-chemical methods, and a figure as low as 5·6 by Rous [1925] from intravital staining of voluntary muscle in mice with the minimum disturbance of the living tissues.

The permeability of the muscle fibre to HCO_3^-

In previous communications the principle was demonstrated for frog muscle (and it has also been found for gland tissue as will be described later) that *the cell membrane in general is permeable both to cations and anions but there are size limits for these ions.* Na, Mg and Ca are excluded as ions, though they obtain entrance into cells, probably in unionized organic combination. On the other hand the muscle is not permeable to the larger anions, such as those of the phosphate esters, but is freely permeable to chloride and very probably to HCO_3^-. The permeability to HCO_3^- throws open to any particular group of cells the whole HCO_3^- buffering of the internal medium. The evidence brought forward by Wallace & Hastings [1942] and Wallace & Lowry [1942] for the impermeability of the muscle membrane may now be considered.

(*a*) Working with the leg muscles of cats they state, 'the intracellular bicarbonate remains relatively unchanged despite wide changes in the extracellular bicarbonate and it is concluded that the muscle cell is normally impermeable to the bicarbonate ion'.

With the membrane *permeable* to HCO_3^- the concentration of this ion in the fibre water is only about 1 mM./kg. Doubling the external concentration outside (other things being equal) only raises it to approximately 2 mM./kg., or from 0·7 to 1·4 mM./kg. whole muscle. The increase due to the interspace water will be at the same time about 2 mM./kg., so that altogether the total CO_2 will increase about 3 mM./kg., which corresponds almost exactly with the mean results described.

(*b*) The evidence advanced from experiments described in a subsequent paper by Wallace & Lowry [1942] is essentially similar in type. In these experiments the abdominal muscle of the rat was equilibrated *in vitro* with solutions containing very varying amounts of HCO_3^- and CO_2 with little variation in the CO_2 tension. They state, 'when the CO_2 pressure remained constant and the bicarbonate ion concentration in the equilibrium fluid was increased from 0·0 to 8·7 mM. per litre, the intraçellular bicarbonate ion concentration remained nearly constant'. Seeing that they measured the extracellular fluid by means of chloride data, on the assumption that the muscle membrane was impermeable to this ion, no other result could be expected—the bicarbonate ion in the fibre so calculated should be constant no matter what the external changes—but it should be zero. What was measured as apparent bicarbonate ion was the Ba-soluble fraction of the total CO_2 and it is not surprising from the results in the present paper that this appeared to be largely independent of the external conditions. Such experiments therefore of Wallace *et al.* provide no real evidence for the impermeability of the muscle fibre membrane to HCO_3^-.

SUMMARY

1. The total acid-labile CO_2 in muscle was determined by alkali extraction and subsequent CO_2 determination by a micro-diffusion method. It was found to be 14·8 ± 0·6 mM./kg. for the abdominal muscle of the rat, 10·3 ± 0·4 mM./kg. and 11·4 ± 0·3 mM./kg. for the abdominal muscle of the guinea-pig and rabbit respectively, and 10·6 ± 0·4 mM./kg. for the leg muscle of the rabbit.

2. The barium-soluble fraction of this total CO_2 amounted to 8·0 ± 0·3, 5·8 ± 0·5, 5·2 ± 0·5 and 7·0 ± 0·5 mM./kg. for the abdominal muscle of the rat, guinea-pig and rabbit and the leg muscle of the rabbit respectively. (The ± values give the S.D. of means.)

3. In the method for determining the barium-soluble fraction it was shown that no appreciable amount of $BaCO_3$ was held in fine suspension or protected from precipitation by proteins from the fact that HCO_3^-—as $KHCO_3$—added to the alkaline extract was precipitated quantitatively; also by turbidity studies. The latter were most satisfactory with abdominal muscle or leg muscle of rabbits of about 0·5 kg. body weight.

4. Allowing for the small amount of free carbon dioxide in the barium-insoluble fraction, it is shown that the ratio of bicarbonate concentration in muscle to that in plasma is the same as for chloride.

5. The pH inside the muscle fibre as determined by the Henderson-Hasselbalch equation applied to the bicarbonate system is 6·0.

6. The pH of the muscle fibre determined from the Donnan principle applied to a membrane permeable to K^+, H^+, Cl^- and HCO_3^- is likewise 6·0.

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THE ACTION OF ADRENALINE AND OF CHOLINE-ESTERS ON THE UTERUS OF THE SHEEP

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When the uteri of different species of animals are compared with regard to their responses to adrenaline, surprising differences are manifested for which it is difficult to suggest any evolutionary causation or any present functional value. Those differences are more conspicuous if the state of the uterus in regard to pregnancy is taken into account.

Langley [1901] showed that adrenaline had a motor effect on the uterus of the rabbit whether pregnant or non-pregnant. Later, Cushny [1906] and Dale [1906] independently showed that, in the cat, the uterus responds differently to adrenaline or to sympathetic stimulation according to its functional condition, the pregnant uterus responding to either of these conditions by contraction, the non-pregnant uterus by relaxation or inhibition.

In the rat Gunn & Gunn [1914] described a third type of innervation, for in this animal the uterus is inhibited by adrenaline both when in the pregnant and non-pregnant condition. They found the same type of innervation in the guinea-pig. Sugimoto [1913] had found that adrenaline inhibited the isolated pregnant uterus of the guinea-pig, but that, with the pregnant uterus *in situ*, intravenous injection of adrenaline produced contraction. Gunn & Gunn, however, found that, both in the rat and guinea-pig, adrenaline relaxed the pregnant uterus whether the organ was exsected or *in situ*.

There are, therefore, three known types of uterine response to adrenaline, exemplified by the rabbit, cat and rat as shown in Table 1. A fourth possibility remained, however unlikely it might be, namely, a uterus which would be stimulated by adrenaline when non-pregnant and inhibited when pregnant. The main object of this paper is to show that such a type of response to adrenaline occurs in the uterus of the sheep. A search of the literature failed

Table 1. Action of adrenaline

Animal	Non-pregnant uterus	Pregnant uterus
Rabbit	Motor	Motor
Cat	Inhibitor	Motor
Rat	Inhibitor	Inhibitor
Sheep	Motor	Inhibitor

to discover any previous investigation of the responses of the uterus of the sheep to adrenaline. Indeed the sheep's uterus has rarely been used for any pharmacological investigation.

METHODS

All experiments were done on the exsected uterus. The apparatus used was of the usual type employed for isolated organs.

The glass cylinder containing the uterus measured 50 c.c., and was fed through a coiled narrow glass tube of a capacity of over 100 c.c., also contained within the copper water-jacket, so that the uterus could be twice washed without any change of temperature of the Locke's solution. Standard Locke's solution without glucose was used. In some later experiments a modification devised by Dr Scott Russell in this department was used, in which a larger cylinder of 100 c.c. capacity was employed and the glass rod used to fix one end of the uterine strip had two hooks at right angles by means of which there could be recorded the contractions and responses of two strips, which, being contained within the same cylinder immersed at the same time, were throughout the experiment under identical conditions as regards time, temperature, nutrient solution, oxygen, etc.

MATERIAL

Four non-pregnant and four pregnant uteri were examined.

The non-pregnant uteri were obtained from the slaughter-house. The organs were removed immediately after the animals were killed, and conveyed to the laboratory in a small thermos pitcher containing cooled Locke's solution. The pregnant uteri were obtained from animals at full-term which were being used for other experiments in this department.

Strips, measuring about 1 × ¼ in. were cut from the uterus. Some strips were tested forthwith. Parts of the uterus, reserved for later examination, were kept in the cold store and strips tested on following days. No qualitative changes in response were found in uteri so kept for up to 4 days.

RESULTS

Action of adrenaline

Non-pregnant uterus. The non-pregnant sheep's uterus is stimulated by adrenaline, as shown by a rise of tone, with, usually, an increased frequency of the individual contractions. A typical response induced by adrenaline, 1 in 5×10^6, is shown in Fig. 1. Of the four non-pregnant uteri examined, one was known to be a virgin uterus. Segments of the body of this uterus were definitely less sensitive to adrenaline than segments of a uterus which was known to have been previously parous. The latter gave pronounced motor response with 1 in 1×10^7 and definite though less marked response with 1 in 5×10^7.

Examination of a large number of segments from four non-pregnant uteri suggests the following conclusions: The response of the exsected non-pregnant sheep's uterus to adrenaline is invariably motor; no segment showed an inhibitory response. This motor response is always elicited by solutions of 1 in 1×10^6 or by stronger solutions. The lowest dilution to give a definite response is 1 in 5×10^7. Weaker solutions have no effect. A parous non-pregnant uterus seems to be more sensitive to this action than a virgin uterus, and the Fallopian tube than the body of the uterus.

Pregnant uterus. Four uteri were examined, all of them from sheep due for normal parturition within a day of the removal of the uterus.

In its response to adrenaline, the pregnant uterus of the sheep shows a sharp contrast to the non-pregnant uterus, as it is invariably inhibited by adrenaline. A typical result is shown in Fig. 2, where the tone of the muscle was relaxed and the contractions completely inhibited by a solution of adrenaline 1 in 5×10^8. All ranges of stronger solutions display this inhibitory effect. With some segments a complete, but transient, inhibition was obtained with solutions so weak as 1 in 2×10^9. The pregnant sheep's uterus is, therefore, as compared with smooth muscle in other organs, one of the most sensitive to the action of adrenaline.

Fig. 1.

Fig. 2.

Fig. 1. (In all tracings contraction of the uterus is recorded by the upstroke of the lever.) Non-pregnant uterus. At *A*, adrenaline, 1 in 5×10^6, showing motor effect of adrenaline.

Fig. 2. Pregnant uterus. At *A*, adrenaline, 1 in 5×10^8. Showing inhibitor effect of adrenaline, with rapid recovery on replacement by Locke's solution at *L.S.*

An analysis of the tests made on a large number of uterine segments with different concentrations of adrenaline showed that the pregnant uterus responds to much lower concentrations than the non-pregnant uterus.

Only uteri at full term have so far been available for investigation. In the meantime it is, therefore, not possible to say at what stage of pregnancy the response of the uterus of the sheep changes from a motor to an inhibitory one.

Fig. 3 shows the responses of a pregnant and non-pregnant uterus, both contained in the same glass cylinder in Scott Russell's arrangement. Adrenaline, 1 in 1×10^6, produced a complete inhibition of the movements of the pregnant uterus and a sustained contraction of the non-pregnant uterus. This tracing shows that the inhibitory action of adrenaline on the non-pregnant uterus is not confined to low dilutions; also that the differences in the responses to adrenaline of the non-pregnant and pregnant uteri are not due to differences in the environmental solutions because in this case both uteri had been throughout the experiment under the same conditions as regards Locke's solution, temperature, oxygen, etc.

Fig. 3. Upper tracing = pregnant uterus; lower tracing = non-pregnant uterus. Both strips in the same container. At *A*, adrenaline 1 in 1×10^6. Showing inhibitory effect on the pregnant and motor effect on the non-pregnant uterus.

Action of some amines related to adrenaline

Ephedrine. The actions of ephedrine were qualitatively similar to those of adrenaline. The non-pregnant uterus was stimulated and the pregnant uterus inhibited. An example of the latter effect is shown in Fig. 4, *E*.

Tyramine (*p*-hydroxy-phenylethylamine) and *β-phenylethylamine* differed from adrenaline in stimulating the pregnant uterus. This action was, however, feeble even with a solution of 1 in 2×10^4. The difference in action on the part of these amines, whereby they stimulate a uterus which is inhibited by adrenaline, recalls a similar difference observed with some other derivatives of phenyl-ethylamine which stimulate the uteri of the rat and guinea-pig though these are inhibited by adrenaline [Gunn, 1939].

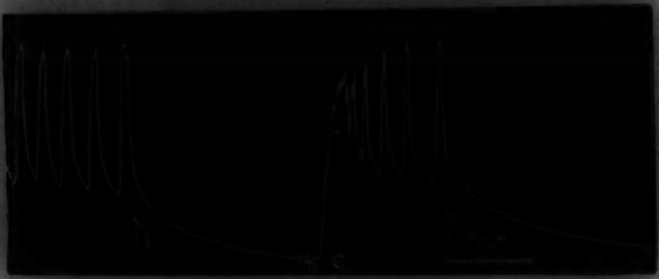

Fig. 4. Pregnant uterus. Showing inhibitor effect of ephedrine, 1 in 5×10^4 at *E*; motor effect of carbachol, 1 in 5×10^4 at *C*, removed by atropine 1 in 1×10^6 at *Atr*.

Action of some choline-esters

Stimulation of the pelvic nerve has a feeble and uncertain motor effect, if any, on the uterus; nevertheless, most uteri react to acetylcholine and any effect so produced is a motor one. Thus Agar [1940] found that, in the exsected uterus of the sexually immature guinea-pig which is inhibited by adrenaline, a concentration of acetylcholine of 1 in 2×10^7 to 1 in 5×10^6 produced a large contraction.

Though the primary object of this investigation was to determine the responses of the sheep's uterus to adrenaline, some experiments were also made to establish the nature of the responses to cholinergic stimulation. These experiments were sufficient to show that acetylcholine produces contraction of the sheep's uterus, whether pregnant or non-pregnant. There seems to be greater differences in sensitiveness of different individual strips, especially of the pregnant uterus, to acetylcholine than to adrenaline, some strips responding to 1 in 1×10^8, whereas others required a concentration of nearer 1 in 1×10^6. An example of the latter finding is illustrated in Fig. 5, where acetylcholine 1 in 1×10^7 produced no marked effect, but 1 in 1×10^6 produced a rise of tone

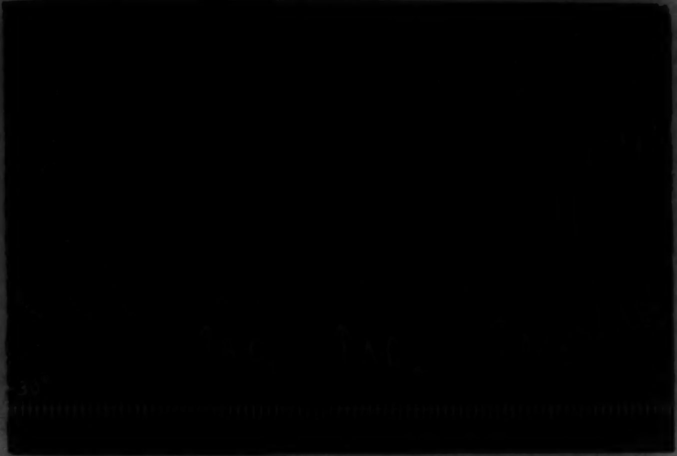

Fig. 5. Pregnant uterus. Showing almost no effect of acetylcholine 1 in 1×10^7 at $A\text{-}C_1$, motor effect of acetylcholine 1 in 1×10^6 at $A\text{-}C_2$, and inhibitory effect of adrenaline 1 in 1×10^9 at *Adr.*

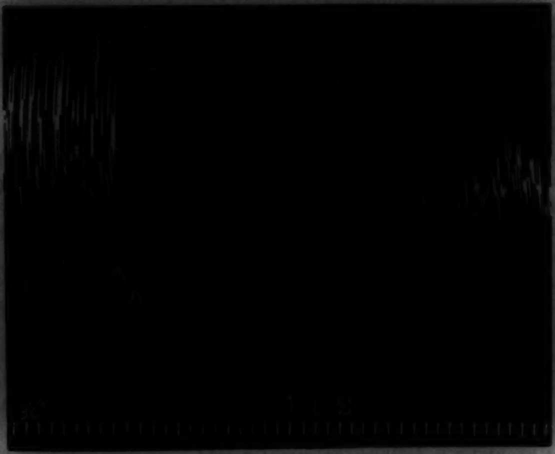

Fig. 6. Pregnant goat's uterus. Showing inhibitor effect of adrenaline 1 in 5×10^8 at *A*, with rapid recovery on replacement by Locke's solution at *L.S.*

and increased frequency of contractions. This particular segment was, however, normally sensitive to adrenaline which caused, in a concentration of 1 in 1×10^9, complete inhibition of the uterine movements.

Carbachol (carbamylcholine chloride), as was to be expected from its known resemblance to acetylcholine, also stimulated both pregnant and non-pregnant uteri. Fig. 4 shows the powerful motor effect of carbachol, 1 in 5×10^4, on the contractions of a pregnant uterus, previously inhibited by ephedrine.

Action of adrenaline on the pregnant uterus of the goat

The opportunity arose of examining the response to adrenaline of segments of the exsected uterus removed from a goat at full term of pregnancy. Adrenaline produced complete inhibition in a concentration of 1 in 5×10^8 (Fig. 6), and definite relaxation of the uterus was produced in other segments by a concentration so low as 1 in 2×10^9. So far as can be gathered from experiments on one animal, therefore, the response of the pregnant uterus of the goat is identical with that of the sheep. The non-pregnant uterus of the goat has not yet been examined.

SUMMARY

Experiments were made on the exsected uteri of four non-pregnant and four pregnant (full-term) sheep.

1. Adrenaline stimulates the contractions of the non-pregnant uterus, but inhibits those of the pregnant uterus. The uterus of the sheep shows a type of response to adrenaline differing from that hitherto described in any species of animal. The pregnant uterus responds to much lower concentrations of adrenaline than does the non-pregnant uterus and, as it may react to a dilution so low as 1 in 2×10^9, is one of the most sensitive of all forms of smooth muscle to the action of adrenaline.

2. Ephedrine acts qualitatively like adrenaline in contracting the non-pregnant, and inhibiting the pregnant, uterus. Tyramine and β-phenylethylamine feebly contract the uterus both in the pregnant and non-pregnant condition.

3. Acetylcholine and carbachol stimulate the contractions of both pregnant and non-pregnant uteri.

4. The exsected uterus of one pregnant (full-term) goat was examined and its response to adrenaline was found to be qualitatively and quantitatively similar to that of the uterus of the pregnant sheep.

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LIBERATION OF HISTAMINE DURING REACTIVE HYPERAEMIA AND MUSCLE CONTRACTION IN MAN

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Barsoum & Gaddum [1935*a*], working on dogs, found that the histamine equivalent of venous blood collected from a limb during reactive hyperaemia increases above normal; it reaches a maximum immediately on release of the circulation after which it rapidly declines. Anrep & Barsoum [1935] obtained a similar effect by severely restricting the arterial blood supply to the gastrocnemius muscle without completely arresting the circulation. Barsoum & Smirk [1936], repeating these experiments on man, observed a two- to four-fold increase of the histamine concentration in the plasma after a 10–20 min. obstruction of the circulation of the arm, the histamine content of the blood corpuscles remaining in most experiments unaltered. The authors conclude that liberation of a histamine-like substance during circulatory arrest accounts at least in part for the hyperaemia. Marcou, Comsa & Chiriceano [1937] found a small increase of histamine in venous blood during the first minute after release of an arterial obstruction of a limb in man.

Recently, Kwiatkowski [1941], also working on man, failed to obtain any evidence of histamine release during reactive hyperaemia; he found no change in the histamine content either of the plasma or of the corpuscles. In view of the importance of this problem for the understanding of the nature of reactive hyperaemia we undertook to repeat these experiments with the object of finding the cause of the conflicting results obtained by the previous observers.

METHODS

Our experiments were made on normal subjects or on patients suffering from minor surgical disabilities. The preparation of the blood extracts and the estimation of histamine were made by the method of Barsoum & Gaddum [1935*b*]. To separate the plasma, the blood was either heparinized or collected in paraffined syringes and centrifuged in cooled paraffined tubes.

The subject was made to rest for about half an hour before the experiment. After withdrawing one or two samples of venous blood from one arm, to serve as a control, a sphygmomanometer cuff was placed round the other arm and inflated to above the systolic pressure for 10–20 min. The needle of the syringe was inserted into the vein a few seconds *before* the release of the circulation, and the first blood sample was collected as exactly as possible between the 5th and 30th sec. after the release. In many experiments a second and a third sample was collected between the 60th and 90th and between the 120th and 150th sec. respectively.

Most previous observers collected the blood samples after releasing the pressure in the cuff to zero. Kwiatkowski, in some experiments, released it to 40 or 60 mm. Hg. This was done in order to prevent the blood from leaving the limb during the period of reactive hyperaemia and to avoid the excessive dilution of the blood which had been in contact with the tissues during the circulatory arrest. Since no evidence was provided that this object was achieved we decided to investigate in preliminary experiments on animals the effect of venous compression upon the extent and duration of the reactive hyperaemia.

Effect of partial venous compression upon the reactive hyperaemia. The experiments were made on dogs anaesthetized with chloralose and injected with chloralose-fast pink (0·1 g./kg.) to prevent coagulation. The femoral artery and vein were dissected for a length of about 3 cm., and a steel band 0·5 cm. in width was passed round the leg under the blood vessels. A cannula was inserted into the peripheral end of the femoral vein after which the steel band was tightened so as to obstruct all the collateral communications. The emerging blood was made to pass through an artificial resistance of the type used in the heart-lung preparation and to enter a reservoir the top of which was connected with a volume recorder. The reservoir was periodically emptied of blood which was then reinjected into the animal. A manometer inserted between the artificial resistance and the leg registered the venous pressure, while the arterial pressure was measured with a mercury manometer which was adapted to record also the maximum-minimum pressures. The records so obtained did not represent the systolic and diastolic pressures of the animal, but the maximal pressure variations including those caused by the respiration.

In order to study the effect which a rise of venous pressure has on the reactive hyperaemia, the resistance on the venous outflow was varied in the different experiments between zero and a pressure midway between the maximal and the minimal arterial blood pressure. The artery was compressed for 10 min. and the venous resistance was adjusted to any desired height about 1 min. before the release of the circulation. Care was taken to prevent the cooling of the limb during the arrest of the circulation. The blood flow during the period of hyperaemia was compared with the resting blood flow as measured with a completely free venous outflow before the artery was compressed. After the decompression of the artery the venous resistance was maintained for not more than 3 min. Only those experiments were considered in which occlusion of the femoral artery led to a complete cessation of the outflow of blood from the vein, showing absence of collateral connexions. The arterial compressions were repeated at intervals of 10–15 min. after the return of the blood flow to normal. The results of a typical experiment are given in Table 1.

TABLE 1. Dog. 9·5 kg. Duration of each arterial compression, 10 min. The maximal-minimal arterial blood pressure was measured shortly before the compression of the artery

Maximal-minimal arterial blood pressure in mm. Hg	Maximal venous pressure in mm. Hg	Normal blood flow in c.c. per 30 sec.	Blood flow during reactive hyperaemia in c.c. in consecutive periods of 30 sec.					
			1	2	3	4	5	6
145/118	0	12·0	79·6	56·1	36·2	26·5	19·9	15·8
145/118	64	11·0	67·1	40·7	27·0	19·5	15·2	14·5
140/116	86	10·5	51·3	35·8	22·6	17·1	13·2	11·2
135/116	106	10·5	28·0	19·6	16·6	13·8	9·4	5·7
132/112	120	10·0	12·9	10·8	7·6	5·1	4·2	3·6

The effect of increased venous resistance upon the hyperaemia was less than expected. Thus, with venous pressures 118, 54, 30 and 10 mm. Hg below the minimal arterial blood pressure the blood flow during the first 30 sec. of the hyperaemia was 6·6, 6·0, 5·0 and 2·7 times respectively above the normal. The increase of the blood flow was abolished only by raising the venous pressure above the minimal arterial pressure.

On applying these results to man, in order to prevent the blood flow from increasing during the initial stages of the hyperaemia above the resting level, the venous pressure should be raised above the diastolic pressure of the subject. Venous pressures of 40–60 mm. Hg, as used by Kwiatkowski, would not be sufficient to prevent the blood from leaving the limb. In view of this conclusion two series of experiments were performed on the human subject. In the first, the blood samples were collected after complete decompression of the sphygmomanometer; in the second, the collection was made after releasing the cuff to a pressure slightly below the systolic pressure.

RESULTS

Observations with complete and partial decompression

The experiments with complete decompression were made on five subjects. The venous blood was collected between the 5th and 30th sec. after release of the sphygmomanometer, while the control blood samples were taken from the other arm either during or shortly before the period of arterial compression. The results of ten observations are given in Table 2.

It can be seen from Table 2 that the histamine equivalent of the plasma collected immediately after the release of the circulation is in every experiment increased above that of the normal plasma. In a few experiments the

TABLE 2. Showing the effect of 20 min. arterial compression on the histamine equivalent of the plasma and corpuscles of the venous blood collected between the 5th and 30th sec. after complete release of the sphygmomanometer. The histamine equivalent is given in μg. of histamine acid phosphate per c.c.

	Histamine equivalent of venous blood in μg./c.c.			
	Normal		Reactive hyperaemia	
Subject	Plasma	Corpuscles	Plasma	Corpuscles
1	0·025	0·13	0·036	0·12
1	0·022	0·12	0·032	0·11
2	0·005	0·10	0·010	0·09
2	0·007	0·08	0·013	0·09
3	0·015	0·13	0·020	0·13
3	0·014	0·12	0·022	0·12
4	0·009	0·14	0·013	0·16
4*	0·007	0·11	0·015	0·13
5	0·008	0·13	0·013	0·14
5*	0·008	0·14	0·016	0·15

* The pressure in the cuff was released in the experiment on subject 4 to 40 mm. and in subject 5 to 60 mm. Hg.

histamine concentration is as much as doubled, in most the increase is less. The results of the two observations in which the cuff was released to 40 and 60 mm. Hg respectively do not differ from the rest.

The experiments with partial decompression were made on four subjects, two of whom had served in the preceding series of observations. After the subject had been allowed to rest and his blood pressure had been measured, his arm was compressed for 10 or 20 min. At the end of the compression the cuff was released to a pressure slightly below the systolic pressure and the blood samples were collected as before between the 5th and 30th sec. after the release. The results of eight observations are given in Table 3.

TABLE 3. Showing the effect of arterial compression on the histamine equivalent of the plasma and corpuscles of the venous blood collected between the 5th and 30th sec. after the release of the sphygmomanometer to a pressure slightly below the systolic pressure of the subject. The histamine equivalent is given in μg. of histamine acid phosphate per c.c. The normal blood samples were collected with a free venous blood flow

				Histamine equivalent of venous blood in μg./c.c.			
				Normal		Reactive hyperaemia	
Subject	Blood pressure in mm. Hg	Pressure in cuff at time of collection in mm. Hg	Duration of compression in min.	Plasma	Corpuscles	Plasma	Corpuscles
3	130/ 85	120	20	0·015	0·14	0·070	0·16
3	130/ 82	120	20	0·013	0·19	0·066	0·18
5	125/ 72	100	20	0·007	0·14	0·028	0·16
5	120/ 70	100	20	0·008	0·12	0·032	0·12
6	150/100	140	10	0·011	0·21	0·022	0·23
6	145/ 95	130	10	0·018	0·24	0·033	0·24
7	115/ 70	95	10	0·013	0·07	0·020	0·08
7	115/ 70	100	20	0·015	0·10	0·067	0·10

It can be seen on comparing Tables 2 and 3 that the increase in the histamine equivalent of the plasma collected with partial decompression after a 10 min.

circulatory arrest is approximately the same as that of the plasma collected with a free venous outflow after a 20 min. compression of the artery. The plasma histamine of the blood collected with partial decompression after 20 min. of arterial occlusion is increased 4–5 times above normal; with complete decompression the increase was less than double. No change in the histamine concentration of the corpuscles was found in the experiments with partial or complete decompression.

The duration of the increased liberation of histamine

In the preceding experiments the blood samples were taken immediately after the end of the arterial occlusion. In order to determine the length of time during which the plasma histamine remains increased above normal, second and third blood samples were collected 1 and 2 min. respectively after the release of the circulation. The results of the experiments performed on four subjects are given in Table 4. Two observations were made on each subject at an interval of a few days; in the first, the blood was collected with complete and, in the second, with partial decompression of the arm.

TABLE 4. Histamine equivalent of the plasma collected at different intervals of time after complete and after partial decompression of the arm. The arterial occlusion was in every experiment 20 min. The histamine equivalent of the corpuscles is omitted from the table as presenting no points of interest. The normal blood samples were collected with a free venous blood flow

Subject	Blood pressure in mm. Hg	Pressure in cuff at time of collection in mm. Hg	Histamine equivalent of plasma in μg./c.c.			
				Reactive hyperaemia		
			Normal	5–30 sec.	60–90 sec.	120–150 sec.
1	—	0	0·024	—	0·022	0·022
	125/85	115	0·022	—	0·040	0·032
2	—	0	0·005	0·009	0·005	0·005
	115/70	105	0·005	0·029	0·012	0·008
3	—	0	0·012	0·018	—	0·011
	135/85	125	0·009	0·048	0·018	—
7	—	0	0·015	0·028	0·016	—
	120/75	105	0·013	0·055	—	0·017

A comparison of the results obtained on each subject shows that with a free circulation no increase in the plasma histamine can be detected in samples collected 1 min. after decompression of the arm. On the other hand, when the venous outflow is restricted the plasma histamine remains above normal for as long as 2 min. after the release of the artery. Similar restrictions of the venous blood flow for 3–4 min. not preceded by a period of arterial occlusion cause no change in the histamine concentration of the plasma.

It follows from these experiments that the histamine produced by the tissues during the period of ischaemia is rapidly washed out unless the blood flow is greatly restricted. With a free circulation the plasma histamine returns to normal long before the end of the hyperaemia. This suggests that the excess histamine appearing in the blood diffuses from the tissues into the capillaries during the period of the circulatory arrest and that probably no further diffusion takes place after the re-establishment of the circulation.

Liberation of histamine during muscular contraction

Having obtained evidence of histamine release during reactive hyperaemia in man we proceeded to investigate if a similar release can be detected in the human subject during muscular contractions. The experiments were made with the same technique as those on the reactive hyperaemia, except that the arrest of the circulation in the arm was continued for only 2 min. The sphygmomanometer cuff was inflated to above the systolic pressure and the subject was told to make fifteen vigorous contractions of his hand and forearm at the rate of one contraction each 2 sec. The contractions were timed to begin on the 60th sec. of the arterial compression and to end 30 sec. later. At the end of the 2 min., i.e. 30 sec. after the last contraction, the pressure in the cuff was released to slightly below the systolic pressure of the subject, and the venous sample was collected in the usual way between the 5th and 30th sec. after the decompression of the artery. The same procedure was followed in the control experiments, which were usually performed on another day, except that the arm remained completely relaxed. The results obtained on two subjects are given in Table 5.

TABLE 5. Showing the effect of arterial compression for 2 min. with the arm at complete rest and with fifteen vigorous contractions made between the 60th and 90th sec. of compression. The normal blood samples were collected with a free venous blood flow

Subject	Blood pressure in mm. Hg	Pressure in cuff at time of collection in mm. Hg	Histamine equivalent in μg./c.c.			
			Normal		Active	
			Plasma	Corpuscles	Plasma	Corpuscles
3 arm at rest	130/ 90	120	0·015	0·140	0·013	0·140
	125/ 82	115	0·012	0·120	0·014	0·120
3 15 contractions	140/105	130	0·014	0·140	0·060	0·130
	130/ 90	120	0·012	0·130	0·085	0·140
8 arm at rest	108/ 68	95	0·010	0·025	0·011	0·022
	110/ 70	95	0·009	0·029	0·010	0·029
8 15 contractions	110/ 70	95	0·012	0·030	0·075	0·070
	110/ 70	95	0·010	0·028	0·050	0·060

It can be seen from Table 5 that when the arm is at rest occlusions of the circulation for 2 min. were insufficient to bring about a detectable change in the histamine equivalent of the venous blood. Similar occlusions of the circulation during which fifteen contractions of the muscles were made by the subject led to a conspicuous increase of the histamine equivalent of the plasma in subject 3, and of the plasma and corpuscles in subject 8. The increase was even greater than that which follows occlusion of the artery for 20 min. with the arm at rest.

Subject 8 presents a special interest as being the only subject in whom the corpuscular histamine increased above normal in most experiments with muscular contraction and arterial occlusion. An explanation of this apparent exception is provided by the observations of Anrep, Barsoum, Talaat & Wieninger [1939*a*], who showed that histamine added to shed blood is not taken up by the corpuscles unless its concentration in the plasma exceeds that of the corpuscles. When this occurs the histamine becomes equally distributed between plasma and corpuscles. Similar conditions obtained in subject 8. His corpuscular histamine was so low that after muscular contractions the plasma histamine increased above that of the corpuscles. As in experiments *in vitro* the excess histamine became equally distributed between the two phases of the blood.

Our observations are in accord with the results obtained on animals which show that the increase of the blood flow [Lewis & Grant, 1926] and the increase of the histamine concentration in the venous blood are much more conspicuous during muscular contractions than after occlusion of the artery [Anrep, Barsoum, Talaat & Wieninger, 1939*b*].

Effect of venous congestion

Reactive hyperaemia is evoked not only by a temporary occlusion of the artery but also by prolonged venous congestion. With the latter, the hyperaemia is shorter and less conspicuous than that following a complete arrest of the circulation of equal duration. The cause of the hyperaemia is undoubtedly the same in both conditions, namely, a reduction or arrest of the blood supply, the changes in the venous pressure being only of incidental interest [Lewis & Grant, 1926]. Anrep & Barsoum [1935] found that diminution of the blood flow in a muscle to about half of the normal is accompanied by a steady liberation of small amounts of histamine. Table 6 shows that venous congestion, if considerable, evokes a liberation of histamine also in the human subject.

TABLE 6. The sphygmomanometer cuff was inflated to below the diastolic pressure of the subject. The blood samples were taken after 20 min. of compression without releasing the cuff. The two experiments on each subject were made with an interval of a few days. The subjects were the same as in the experiments given in Table 5. The normal blood samples were collected with a free venous blood flow

			Histamine equivalent in μg./c.c.			
			Normal		Venous congestion	
Subject	Blood pressure in mm. Hg	Pressure in cuff in mm. Hg	Plasma	Corpuscles	Plasma	Corpuscles
3	130/90	50	0·007	0·120	0·009	0·130
	135/95	90	0·009	0·100	0·028	0·110
8	110/70	40	0·009	0·029	0·011	0·029
	115/75	70	0·012	0·024	0·036	0·040

A 20 min. compression of the arm with a pressure 30–40 mm. Hg below the diastolic pressure of the subject caused no obvious change in the histamine concentration of the blood. On the other hand, when the compressing pressure was raised almost to the height of the diastolic pressure, the histamine of the plasma increased in both subjects about three times above the normal. As in experiments with muscular contractions the histamine equivalent of the corpuscles of subject 8 increased, to become equal to that of the plasma. Venous congestion of short duration had no detectable effect on the histamine of the blood.

DISCUSSION

The experiments described in this communication confirm and extend our previous observations made on animals. In the human subject, as in the dog, conditions accompanied by a relatively deficient circulation lead to a liberation of measurable amounts of histamine. During the large increase of the blood flow which follows a period of ischaemia the histamine containing blood is greatly diluted with fresh blood and is rapidly washed out of the limb, the histamine equivalent returning to normal within the first minute of the reactive hyperaemia.

The increase in the histamine equivalent of the blood is rendered more conspicuous when the blood flow, during the period of the hyperaemia, is prevented from increasing above normal by compressing the veins with a pressure above the diastolic pressure of the subject. Venous compression with 40–60 mm. Hg, as used by Kwiatkowski, is insufficient since it does not greatly affect the blood flow during the initial stage of the hyperaemia. When the blood flow through the arm is controlled, an increase in the histamine of the plasma can be found as late as 2 min. after the onset of the hyperaemia.

The failure of Kwiatkowski's experiments to demonstrate a liberation of histamine during reactive hyperaemia was probably due to an insufficient control of the blood flow through the arm and to the collection of the blood samples at a time when the liberation of histamine had already ceased.

Vigorous muscular contraction of short duration is accompanied in man, as in the dog, by a release of histamine which is considerably larger than that following arterial occlusion of the same duration. There is no reason to suppose that the cause of histamine liberation by the contracting muscle is different from that operating during arterial occlusion or venous congestion. In all these conditions there is a relative local deficiency of circulation. The liberated histamine must be considered as one of the factors which readjust the balance between the metabolic requirements of the tissues and their blood supply.

SUMMARY

1. Muscular contraction, arterial occlusion and prolonged venous congestion in the human subject are accompanied by a release of histamine by the tissues.

2. The release of histamine is rendered more conspicuous by controlling the circulation during the period of the hyperaemia by raising the venous pressure above the diastolic pressure of the subject.

3. In most subjects the histamine increases only in the plasma; in subjects with a low histamine equivalent of the corpuscles the histamine increases in plasma and corpuscles.

4. The reasons of Kwiatkowski's failure to demonstrate a liberation of histamine during reactive hyperaemia are discussed.

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EMULSIFICATION OF FAT IN THE INTESTINE OF THE RAT AND ITS RELATIONSHIP TO ABSORPTION

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Until the end of the last century it was generally accepted that fat was absorbed from the intestine in a state of fine division without previous hydrolysis. Pflüger [1900] opposed this view and suggested that hydrolysis was an essential preliminary to absorption and that the fatty material was absorbed in a state of molecular dispersion as soap. The necessity for hydrolysis is still maintained by Verzar & McDougall [1936], who regard the absorption of unhydrolysed triglyceride as an impossibility. They, however, in putting forward a lipolytic hypothesis do not accept saponification as a mechanism of absorption of fatty acid, but postulate the formation of a diffusible complex of fatty acid with bile acid. It has been shown by Frazer [1943*a*] that fat and fatty acid behave differently during and after absorption, and that lipolysis may be a determining factor in the destination and fate of absorbed fatty material [Frazer, 1943*b*]. These findings have been explained by the partition hypothesis, according to which triglyceride is only partly hydrolysed prior to absorption. The object of this communication is to put forward evidence which indicates a possible mechanism of absorption of unhydrolysed triglyceride

METHODS AND MATERIALS

Animals. Throughout these experiments, adult rats from our own breeding stock were used.

Materials. The chemicals used in these experiments were olive oil B.P., oleic acid (redistilled), liquid paraffin B.P., sodium oleate (B.D.H.), pure anhydrous sodium carbonate, sodium glycotaurocholate (B.D.H.), and glyceryl monostearate.

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Emulsification. The emulsions used in these experiments were prepared with the apparatus described by Frazer & Walsh [1933]. The dispersion was very uniform, the average particle diameter being less than 0·5 μ. The details of their composition are fully described in the text.

The examination of possible emulsifying systems was made by simple test-tube experiments using paraffin or olive oil as the dispersed and buffer solutions as the continuous phase. The substances under investigation were added to the appropriate phase before mixing.

The criteria applied in the assessment of a satisfactory emulsifying system for intestinal absorption were:

(i) The occurrence of spontaneous emulsification on mixing without agitation.

(ii) The formation of fat globules averaging 0·5 μ or less in diameter after 20 shakes.

(iii) Stability of the emulsion over a period of 3 hr.

The size and stability of the resulting emulsions were checked in each case by microscopical study with dark-ground illumination. The *p*H determinations were made throughout these experiments with the glass electrode, and KH_2PO_4/NaOH buffer was used.

Experimental procedure. The animals were fed whenever possible intragastrically. Emulsions stabilized with sodium oleate cannot be injected into the stomach as the acid causes breaking of the emulsion. Such emulsions were injected directly into the duodenum, and for quantitative studies pyloric and ileal ligatures were used. These ligatures were applied with great care to avoid any unnecessary manipulation of the intestine, and from control experiments it was found that this procedure did not cause any gross interference with absorption during the experimental period. In all cases the animals were starved for 24 hr. before the experiment was begun and, if an anaesthetic was required, ether was used. The animals rapidly recovered from the anaesthetic and the operative procedure and appeared quite normal throughout the experimental period. The amount of absorption in each case was determined by analysing the residual contents of the intestine. This was done by connecting the down arm of a reflux condenser with the intestine, so that the alcohol-ether mixture ran through the lumen of the gut. In addition to residual analysis, the absorption was checked by frozen sections of intestinal cells.

RESULTS

Emulsification in the intestine

If 1 c.c. olive oil was administered intragastrically to a rat and the animal killed 3–4 hr. later, the fat in the small intestine was found to be in a very finely divided state. The size of the particles was less than 0·5 μ diameter, and the emulsion appeared to be stable for several hours. If, on the other hand, paraffin is administered to a rat, emulsification does not occur and the globules vary in size from about 10μ up to large masses of free oil. The fine emulsification of olive oil has been observed in more than 100 rats, and gives rise to the characteristic white appearance of the intestine in animals killed 3–6 hr. after ingestion of olive oil.

Absorption of emulsified paraffin from the intestine

The following emulsions were prepared:

(*a*) 50% paraffin emulsion stabilized with equimolecular amounts of cholesterol and sodium oleate, the latter being formed *in situ* at the oil/water interface. The final oleate percentage was 2% and the average particle diameter was about 0·5μ.

(*b*) 50% olive oil emulsion stabilized as in (*a*) and having the same degree of dispersion and stability.

(*c*) 50% paraffin emulsion stabilized as in (*a*), but not so finely dispersed. This emulsion contained many particles of 1–2μ or more in diameter.

(*d*) 50% paraffin emulsion stabilized with equimolecular amounts of cholesterol and sodium cetyl sulphate. The concentration of sodium cetyl sulphate was 2%. The average particle diameter was less than 0·5μ.

In this group of experiments 120 rats were used. The emulsions were administered by intraduodenal injection, and the amount of absorption in each rat was determined by residual analysis after 6 hr. The absorption was also checked histologically in a number of cases. Unemulsified paraffin was injected in one control group. The average results compiled from each group of experiments are shown in Table 1.

TABLE 1. Absorption of emulsified paraffin from the intestine

Material injected	No. of experiments	Amount absorbed mg.	Percentage of oil or paraffin given which was absorbed
Fine paraffin emulsion (*a*)	50	163	36
Fine olive oil emulsion (*b*)	30	172	33
Coarser paraffin emulsion (*c*)	15	73	11
Finer paraffin emulsion (*d*) (cetyl sulphate)	15	228	43
Unemulsified paraffin	10	Nil*	Nil*

These figures represent the average obtained in the number of individual experiments quoted. In every series of experiments an emulsion aliquot was analysed to check the amount of oil or paraffin administered. The experimental period in all cases was 6 hr.

* The amount recovered corresponds in the unemulsified paraffin group with the amount administered within the limits of an experimental error of ±5%.

In vitro *experiments on emulsification*

The object of this group of experiments was to investigate the possible emulsifying systems that might be effective in the intestine. The substances studied include bile salts, fatty acid, soap, cholesterol and monoglyceride. The interrelation between these possible factors and the effects of change of the *p*H of the continuous phase was also studied.

The *p*H of the contents of the upper part of the small intestine is said to be 6·5 [Kostyal, 1926; Robinson, 1935]. In a series of experiments, using eighty rats and determining the *p*H with a capillary glass electrode, we found that this was the usual reaction of the intestinal contents in rats just after killing with coal gas. If precautions were taken to prevent the rapid passage of stomach contents into the duodenum at the time of death, rather higher readings were obtained, but they were not particularly significant in the problems of emulsification, as the reaction may certainly be more acid than *p*H 7·5. From our experiments, it appeared that the *p*H of the intestinal contents varied over a range from 6·5 to 8·0. We have never found a more acid reaction than *p*H 6·3. We have, therefore, studied the emulsifying systems over a *p*H range of 6·0–8·5, which adequately covers the physiological range in the small intestine. The *p*H of all mixtures was checked by glass electrode at intervals throughout the experiments.

(1) *Single factors*

Bile salts. 1% bile salts lowered surface tension to some extent, but they were found to be poor emulsifying agents throughout the *p*H range of 6–8·5. There was no spontaneous emulsification and the average particle size was greater than 2μ. The stability was very poor, and the emulsion started to break immediately. Increase of concentration above 1% caused further deterioration in emulsifying action.

Fatty acid and soap. Soap causes a marked decrease in interfacial tension, especially if fatty acid is in the oil phase and alkali in the water phase [Donnan, 1899]. A considerable difference in emulsifying properties was found between preformed soap and soap formed *in situ* at the oil/water interface. Thus if oil was added to a 1% preformed solution of sodium oleate, there was no spontaneous emulsification, and after agitation the particles exceeded 2μ in diameter, and stability was poor. If oleic acid was added to the oil phase and sodium carbonate to the water phase in amounts equivalent to the sodium oleate in the previous experiments, spontaneous emulsification occurred on mixing the two phases. Agitation resulted in the formation of particles with an average diameter of about $0{\cdot}5\mu$ and stability was good.

In studying the effects of change of *p*H in the water phase, soap must be considered with fatty acid, since both exist in varying proportions at different points in the *p*H range. With a *p*H of 8·5 spontaneous emulsification occurred with fine dispersion and good stability, but at more acid *p*H there was no spontaneous emulsification, and dispersion and stability became progressively worse. It was possible to improve emulsification a little by the addition of a large excess of oleic acid, but even so it was not possible to demonstrate satisfactory emulsification at pH 7·5 or less.

Cholesterol. Cholesterol alone had no emulsifying action.

Glyceryl monostearate. Glyceryl monostearate added to the oil phase gave rise to no spontaneous emulsification. On agitation some emulsification occurred, but the particle size was greater than 2μ, and stability was poor. Monoglyceride in the absence of an ionizing agent favours the formation of a water-in-oil emulsion.

(2) *Paired interrelationships*

Bile salts/oleic acid. Bile salts gave some emulsification of oleic acid upon agitation, which was more effective in acid media. The size of the particles was greater than 2μ, and stability was poor. The combination of bile salts with oleic acid did not, however, give any improvement in its emulsifying action on triglyceride or paraffin as compared with the action of oleic acid alone. The effect of pH change was identical with oleic acid alone or with the oleic acid/bile salt complex; emulsification was only effective at pH 8·5.

Soap/cholesterol. If cholesterol was added to the oil phase, more satisfactory emulsification occurred with preformed soap than when this substance was used alone. Both dispersion and stability were much improved. That this was due to the formation of a soap/cholesterol complex was demonstrated by the fact that cholesteryl esters actually inhibit emulsification [Schulman & Cockbain, 1940]. The combination of cholesterol with soap did not, however, significantly alter the deterioration in emulsifying action in more acid pH than 8·5.

Oleic acid/monostearate. With this combination, emulsification was improved, and spontaneous emulsification with fine dispersion occurred at pH 7·5. At a more acid reaction, the emulsifying effect rapidly deteriorated, and the emulsifying effect of oleic acid/monostearate showed no improvement over oleic acid or monostearate alone at pH 6.

Bile salts/monostearate: Bile salts/cholesterol: Cholesterol/monostearate. With these various combinations no change or improvement in emulsifying action can be demonstrated over the effects obtained with the substances used singly.

(3) *Triple interrelationships*

Bile salts/oleic acid/glyceryl monostearate. If oleic acid and monoglyceride were dissolved in the oil phase, and bile salts were added to the water phase, mixing resulted in spontaneous emulsification throughout the pH range 6–8·5. The particle diameter averaged less than $0{\cdot}5\mu$, and stability was excellent. The emulsification deteriorated if the concentration of bile salts was increased above 1%, and it improved with increasing concentrations of oleic acid and monoglyceride up to 20 and 12% of the oil phase respectively. The possible significance of these concentrations is being further investigated.

Bile acid/oleic acid/cholesterol and oleic acid/cholesterol/monoglyceride. These combinations showed no improvement upon the effects obtained with the corresponding paired substances. The experiments on emulsifying systems are summarized in Table 2.

TABLE 2. A comparative study of possible intestinal emulsifying systems

Emulsifying systems *p*H	6·0	6·5	7·0	7·5	8·0	8·5
1. Bile salts	–	–	–	–	+*	+*
2. Oleic acid (soap)	–	–	–	+†	++	+++
3. Monostearate	–	–	–	–	+	+
4. Cholesterol	–	–	–	–	–	–
5. Bile salt/oleic acid	–	–	–	+	++	+++
6. Oleic acid/monostearate	–	–	+	++	++	+++
7. Soap/cholesterol	–	–	–	+	++	+++
8. Bile salts/monostearate	–	–	–	–	++*	++*
9. Bile salts/cholesterol	–	–	–	–	+*	+*
10. Cholesterol/monostearate	–	–	+	+	+	+
11. Oleic acid/bile salts/monostearate	+++	+++	+++	+++	+++	+++
12. Bile salt/oleic acid/cholesterol	–	–	–	+	++	+++
13. Oleic acid/cholesterol/monoglyceride	–	–	+	++	++	+++

– No emulsification.
+ Emulsification on agitation: particles average more than 2μ.
+ + Some spontaneous emulsification: particles on agitation exceed an average of 1μ.
+ + + Spontaneous emulsification: dispersion of particles less than 0·5μ average.
* Effect probably due to soap impurities.
† Effect obtained with excess oleic acid.

DISCUSSION

If unhydrolysed fat is to be absorbed from the intestine, it must pass through the membrane of the intestinal cell either in a state of molecular dispersion, as a diffusible complex, or in a state of fine emulsification. With regard to the two former methods of absorption, there is at present no evidence to suggest that triglyceride fat can be readily dispersed in molecular division in an aqueous medium by any of the agents known to be in the intestine. The molecular absorption of triglyceride by the intestinal cell has also been objected to on theoretical grounds [Davson & Danielli, 1943]. Bile salts form an association with fatty acid, but no complex-forming substance has so far been found which would render triglyceride fat freely diffusible. It seemed probable, therefore, that fine dispersion as an oil-in-water emulsion might be the form in which unhydrolysed triglyceride fat is absorbed.

It is apparent from the first group of experiments that fat is in fact very finely emulsified in the small intestine. If a rat is fed olive oil and killed 4 hr. later, the small intestine is found to be filled with a white milky material which on further examination can be shown to consist of finely dispersed negatively charged globules of olive oil, with an average diameter of less than 0·5μ. The size of these emulsion particles is similar to that of the fat globules seen in the intestinal cell, and of the chylomicrons found in the blood at the height of fat absorption.

The second group of experiments deals with the absorption of paraffin from the intestine. It is well known that paraffins are not readily absorbed. This has been attributed to the fact that the paraffin molecules cannot be changed by lipolysis, and that paraffin does not form a diffusible complex with bile salts. Our experiments indicate that paraffins can be absorbed in amounts comparable with olive oil administered under similar conditions. It is essential, however, that the paraffin should be finely emulsified so that the particles are less than 0·5μ in diameter. There does not appear to be any significant difference between the absorption in a 6 hr. period of finely dispersed negatively charged particles of paraffin or olive oil. The experimental evidence also indicates the importance of the degree of dispersion of the oil phase. It is suggested that the reason for the non-absorption of unemulsified paraffin lies in the fact that paraffin is unable to provide its own emulsifying mechanism. The significance of the lack of hydrolysis of paraffins in this connexion will be more apparent when the nature of the emulsifying mechanism is discussed later in this paper.

The absorption of finely dispersed paraffin emulsion may perhaps be correlated with the findings of Baker [1942], who, working independently, has investigated the structure of the free border of the epithelial cells of the intestine in vertebrates. He finds that the free border consists of three layers: a superficial layer next to the lumen of the intestine, which is pierced by pores; a canal layer containing spindle-shaped canals running at right angles to the surface which are continuous with the pores of the superficial layer; and a granular layer next to the cytoplasm of the cell. The diameter of these canals he estimates to be about 0·5μ. They are well shown in numerous microphotographs in his paper, but are even more convincing in the actual preparations. Such a structure may be of significance in the passage into the intestinal cell of fine fat globules, the diameter of which is known to be rather less than 0·5μ. The various factors which may be concerned in the passage of these charged particles through such canals have yet to be determined.

Having shown that finely dispersed emulsions of an unhydrolysable material can be absorbed, and that a similar fine dispersion of fat occurs in the intestine, the third group of experiments was designed to determine the nature of the emulsifying mechanism involved. This system must be able to produce spontaneous emulsification of oil to the degree of dispersion which we have found necessary for absorption, namely, an average particle diameter of less than 0·5μ. Stability for some hours would also seem to be desirable and the system must be capable of functioning over a pH range of 6–8·5.

The main factors that seemed likely to be of importance were soap, fatty acid, bile salts, monoglyceride, cholesterol and phospholipid. Of these, phospholipid has been excluded from our investigations, since evidence was obtained which indicated that phospholipid was not a significant constituent of the interfacial film of the fat globules in the intestine. It can be demonstrated as a part of the structure of the chylomicron. It is suggested that it may be added to the particle in the intestinal cell [Elkes & Frazer, 1943]. We cannot ignore the possibility of involvement of phospholipid in the emulsifying mechanism, but our evidence at present is against it.

In order to determine the importance or otherwise of the other factors, experiments were carried out in three groups—each substance alone, and in double, and then triple combinations of the factors. Each series was studied over the *p*H range 6–8·5, and the effects of varying concentrations of the factors was determined. The criteria of spontaneous emulsification, particle size and stability were applied to each series.

Of the single factors only oleic acid (soap) gave any satisfactory emulsification, and this was confined to the extreme alkaline end of the *p*H range (*p*H 8·5). The greatest effect on the interfacial tension occurs when the soap in the interfacial film is acid soap. Traces of calcium ions must be considered. At an air/water interface the maximum lowering of interfacial tension with soap coincides with the point of half-dissociation. At the oil/water interface, however, the maximum lowering of interfacial tension with sodium oleate is found to occur in much more alkaline solutions (*p*H 8·5 or higher) owing to the removal of oleic acid from the interfacial film into solution in the oil phase. The effect of this loss of oleic acid from the film can be counteracted to some extent by the addition of excess oleic acid [Powney, 1935; Powney & Addison, 1938]. In our experiments, even with the addition of excess oleic acid, we were unable to obtain emulsification below *p*H 7·5, and at this level the dispersion was not satisfactory. The only other single factor which requires mention is bile salt. It is commonly stated that the bile salts are good emulsifying agents for fat. The interfacial tension between oil and water may be lowered to about 6 dynes/cm. by impure bile salts, and to a much less degree by pure bile salts. Soap will lower the tension to less than 3 dynes/cm., and certain complexes to less than 0·5 dyne/cm., which is the order of interfacial tension required to allow of the fine dispersal of fat required for absorption. It is clear, and can easily be demonstrated, that bile salts alone cannot be regarded in any way as a satisfactory emulsifying agent for fat. Many of the bio-physico-chemical properties commonly attributed to bile salts, such as emulsification of fats and haemolysis [Schulman & Rideal, 1939] can be accounted for by the presence of soap impurities.

Of the paired factors, two form complexes—oleic acid/bile salts and soap/cholesterol. The association between oleic acid and bile salt was first described by Wieland & Sorge [1916] and has been adequately confirmed since. The complex is, however, only very slightly more effective as an emulsifying agent than oleic acid alone. The soap/cholesterol complex does, however, give some improvement in dispersal and stability, but this is not marked if the soap is formed *in situ* at the oil/water interface, since emulsification under these circumstances is already excellent. In any case, the addition of cholesterol does not overcome the effect of change of *p*H towards the acid side on the stabilizing soap film. The combination of oleic acid with monoglyceride also shows a marked improvement over either of these two factors alone. The probable reason for this is that monoglyceride forms an excellent interfacial film due to the hydrophilic glycerol and oleophilic fatty acid portion of its molecule, but it has the disadvantage of being uncharged, which favours the formation of water-in-oil emulsions. The addition of oleic acid to the interfacial film provides a charge due to the ionized COO^- groups. Change of *p*H towards the acid side results in suppression of ionization of the oleic acid with a decrease of charged COO^- groups and an increase of uncharged COOH groups (Fig. 1). Consequently the charge on the particles begins to disappear at about *p*H 7·5 and the emulsifying action rapidly deteriorates.

The triple combination of factors showed one outstanding emulsifying system, bile salt/oleic acid/monoglyceride. This system gave spontaneous emulsification and adequate dispersal and stability over the whole of the *p*H range (6–8·5) and even down to *p*H 4·0. The oleic acid and monoglyceride were dissolved in the oil phase and the bile acid added to the water phase. The probable mechanism involved in this emulsifying system is indicated in Fig. 1. As the *p*H is shifted towards the acid side, the number of COO^- groups decreases and COOH groups increases as described above. Bile salts form an association with unionized oleic acid and so become involved in the interfacial structure. It is suggested that the SO_3^- group in the taurocholate provides the necessary negative charge for continued emulsification and stability when the *p*H is such that oleic acid ionization is seriously depressed. In this way with the triple complex we have an emulsifying system which is entirely independent of any *p*H change which may occur in the intestinal contents.

The provision of this triple combination of bile salts/fatty acid/monoglyceride is a simple matter in the normal intestine. The former is provided in the bile, while the latter two factors can be formed by partial hydrolysis of triglyceride. Furthermore, in the case of lipolysis both fatty acid and monoglyceride are being formed *in situ* at the oil/water interface. Cholesterol forms an ion-dipole complex with ionized soap which will anchor the soap molecule to the oil/water interface and prevent it from diffusing into the water phase, thus stabilizing the interfacial film and the emulsion [Schulman & Cockbain, 1940]. Cholesterol does not appear to be of great significance in our experiments on emulsifying systems, but it may assume greater importance when the fatty acid is gradually accumulating at the interface as in lipolysis. The relationship of cholesterol to soap at the interface may be of importance in the absorption of cholesterol.

On the basis of the experiments reported, it is concluded that finely dispersed particles of fat or paraffins can be absorbed from the intestinal lumen and that suitable emulsification of fat does occur in the intestine. It is also clear that bile salts and partial lipolysis, giving rise to monoglyceride and fatty acid, will provide an effective emulsifying system which is not altered within the *p*H range of 6–8·5. The non-absorption of unemulsified paraffins may be attributed to inability to provide the essential constituents of the emulsifying system. The relationship of these findings to the absorption of fat under normal physiological conditions is being further investigated.

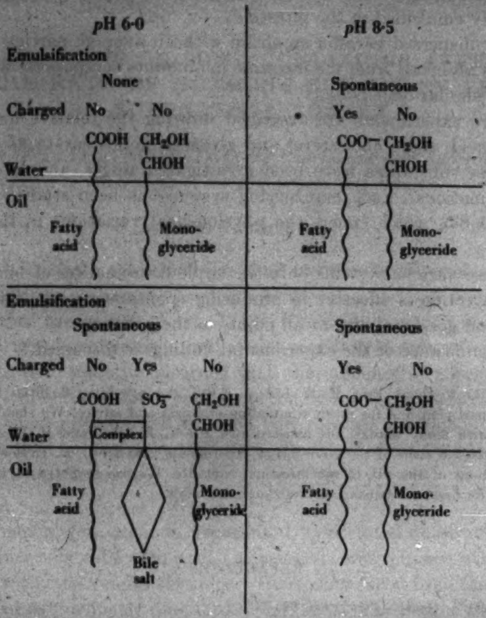
pH 6·0
pH 8·5
Emulsification
None
Spontaneous
Charged No No
Yes No
COOH CH₂OH
CHOH
COO⁻ CH₂OH
CHOH
Water
Oil
Fatty acid
Mono-glyceride
Emulsification
Spontaneous
Spontaneous
Charged No Yes No
Yes No
COOH SO₃⁻ CH₂OH
CHOH
Complex
Bile salt

Fig. 1. Diagram of the suggested arrangement at the oil/water interface in the bile salt/oleic acid/monostearate system. The wavy lines represent the hydrophobic portion of the fatty acid molecule. The diamond-shaped figure indicates the steroid portion of the bile salt molecule. The bile salt molecule has been omitted from the fourth diagram in the series because it is not regarded as an essential feature of the interfacial structure at *p*H 8·5. The diagram shows the importance of the presence of a charged molecule in addition to monoglyceride in the interfacial film.

Summary

1. Ingested triglyceride is finely dispersed when it enters the small intestine. The average diameter of the fat globules is less than $0{\cdot}5\,\mu$. Paraffins are not finely emulsified in the intestine.

2. Finely dispersed paraffin emulsion with an average particle size of less than $0{\cdot}5\,\mu$ is absorbed from the intestine in amounts comparable with olive oil emulsion of similar dispersion.

3. *In vitro* experiments are described showing the relative merits of bile salts, oleic acid, soap, cholesterol and glyceryl monostearate as emulsifying agents. These substances have been investigated singly, and in double and triple combinations. Each emulsifying system has been studied over a pH range of 6·0–8·5, which covers the physiological variations in the small intestine.

4. Of these various systems only the triple combination of bile salts/oleic acid/monoglyceride is effective in producing spontaneous emulsification, fine dispersion and good stability at all points in the pH range.

5. The significance of the experimental findings is discussed.

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OBSERVATIONS ON SOME CONDITIONS AFFECTING THE RATE OF HORMONE OUTPUT BY THE SUPRARENAL CORTEX

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Considerable amounts of cortical hormone are continuously released into the circulation by the suprarenals, and can be detected and assayed in the blood collected from the glands [Vogt, 1943]. The present paper deals with the effect of various factors on the rate of this secretion of cortical hormone. The experiments were mainly concerned with the influence of adrenaline and of splanchnic stimulation. Since both these conditions are liable to cause changes in arterial blood pressure and in blood flow through the glands, preliminary experiments had to ascertain the effect, if any, of these two factors on the output of cortical hormone.

METHODS

The experiments were done on heparinized, eviscerated dogs, except for two instances, in which cats were used. Anaesthesia was induced with ether and completed with intravenous chloralose. The venous blood from the suprarenals was collected as previously described [Vogt, 1943]. The plasma prepared from this blood was assayed for cortical activity by hypodermic injection into groups of suprarenalectomized rats exposed to cold [method of Selye & Schenker, 1938]. The prolongation of the mean survival time of such groups of rats by the administration of plasma measures its content of cortical hormone. Details will be found in the earlier publication.

There are two ways of following the minute output of hormone throughout an experiment. The first is to collect consecutive samples of equal volume. If appreciable changes in blood flow take place, the periods of collection will be of different length and the output per minute will have to be found by calculation. Since the assay is not strictly quantitative, this introduces an error, and the method, accordingly, was only used in a few preliminary experiments. The alternative is to make the collection times equal, irrespective of the volumes of the samples. In this case, the assay measures the output without any corrections. Since, however, injection into the rats of unequal volumes of plasma may lead to errors through treating the groups with varying amounts of fluid and of protein, the volumes of samples appreciably differing in size were rendered equal before injection by the addition of arterial plasma. This was permissible, since it had previously been found that arterial blood does not contain amounts of cortical hormone detectable by biological assay.

Intravenous infusion of 0·9% sodium chloride or heparinized blood was sometimes used to counteract the fall in blood pressure resulting from the loss of blood through the suprarenal veins. The use of blood, however, was severely restricted by the difficulty of obtaining adrenaline-free samples of arterial blood from a recently anaesthetized animal, since the presence of adrenaline, as we shall see later, seriously affects the results.

In all instances in which the suprarenal blood was likely to contain adrenaline, its adrenaline content was assayed on the rabbit's intestine. As has been shown previously [Vogt, 1943], no significant effect on survival time was produced by adding adrenaline to solutions of cortical hormone or to saline and injecting these mixtures into suprarenalectomized rats. Nevertheless, whenever one of several samples contained a considerable quantity of adrenaline, it was thought advisable to equalize the content of all plasma samples by the addition of adrenaline.

Before being assayed on the rats, the plasma was kept in the refrigerator for ½–2½ days.

The effect of adrenaline on cortical secretion was tested by slow intravenous infusion of dilute solutions for periods between 6 and 25 min., the total amount administered ranging between 4·5 and 226 μg./kg.

Electrical stimulation of the splanchnic nerves was performed with a pair of bipolar shielded electrodes, an induction coil with one accumulator in the primary circuit, and Lewis's interrupter producing 10 break shocks per sec.

Close arterial injections of acetylcholine were performed in a manner similar to that described by Feldberg & Minz [1932] on cats. During evisceration, care was taken to leave a sufficient length of the coeliac artery to permit insertion of a cannula; this was connected by a length of pressure tubing bearing a clamp to a large, rigidly fixed syringe containing acetylcholine HCl 1:10,000. One ml. was rapidly administered from the syringe every 15 or 20 sec.

RESULTS

Effect of changes in arterial blood pressure and blood flow through the gland on output of cortical hormone

In previous experiments [Vogt, 1943], no correlation was found between the minute output of cortical hormone and different levels of blood pressure or varying rates of blood flow. Comparison, however, was mainly restricted to the yields of hormone obtained in different animals. It will now be shown that the result is the same if such changes occur in the course of one experiment. Table 1 gives the figures obtained in an experiment on a dog (see also Fig. 1).

TABLE 1. Yields of cortical hormone at varying levels of arterial blood pressure (Exp. 62)

No. of sample ...	1	2	4	5
Mean survival time of group of rats (hr.)	6·77	6·42	6·49	6·39
Average blood pressure (mm. Hg)	80	65	39	68
Blood flow (ml./min.)	2·28	1·80	0·92	1·73

Four samples of blood were collected from the left suprarenal for periods of 15 min., the plasma was separated off, and each plasma assayed for cortical hormone on a group of rats. The mean survival times of the groups are shown in the table: they are practically the same, despite the considerable differences in blood pressure and blood flow during the four periods of collection. The fall in blood pressure from 80 to 39 mm. Hg and the corresponding decrease in flow were due to the draining of blood from the suprarenals. The rise in blood pressure and the acceleration of flow during the last 15 min. were produced by infusion of blood which had been obtained earlier on from the carotid.

In other experiments, identical yields were frequently seen in samples collected from the same animal at intervals of several hours, during which blood pressure and blood flow had fallen considerably. An effect of these two factors on the 'resting' secretion of the suprarenal cortex was never ascertained.

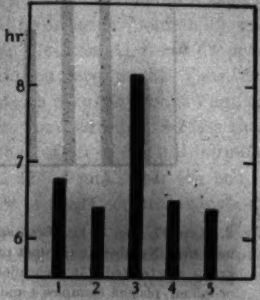

Fig. 1. Exp. 62, dog, 11 kg. Both splanchnic nerves cut. Ordinate: mean survival time of rats in hours. Number at the foot of each column indicates number of sample with which the group was treated. 0·35 ml. plasma administered at each injection. Collection time for all samples 15 min. *Sample* 1 and *Sample* 2: control periods. *Sample* 3: collected during the last 15 min. of an intravenous infusion of 95 μg. adrenaline lasting 20 min. *Sample* 4, 17 min., and *Sample* 5, 34 min. after the end of the adrenaline infusion. 50 ml. of heparinized blood given intravenously just before, and 73 ml. during the collection of *Sample* 5.

Adrenaline infusion

In the early experiments, the splanchnic nerves were cut on both sides shortly before the collection of samples from the lumbar vein was started. When, however, experience had shown that adrenaline has a powerful and long-lasting action on the suprarenal cortex, great care was taken to exclude the after-effects of any adrenaline secreted from the animal's own glands, by severing the splanchnic nerves at the beginning of the operation before performing the evisceration.

Fig. 1 (Exp. 62) is an example of the effect. The heights of the five columns represent the mean survival times resulting from injecting into groups of rats samples of plasma collected for 15 min. each. The first two samples were taken before the adrenaline infusion. Sample 3 was obtained during the last 15 min. of an infusion of a total of 95 μg. adrenaline (8·6 μg./kg.) in a period of 20 min. Its potency (column 3) is significantly increased over that of the previous ones. In the two subsequent samples (columns 4 and 5), the output of cortical hormone has returned to normal. Their collection was started 17 min. (sample 4) and 34 min. (sample 5) after the end of the administration of adrenaline. During the infusion, the average blood pressure was 74 mm. Hg

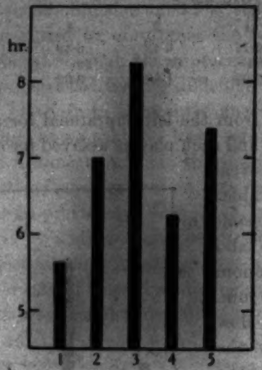

Fig. 2.

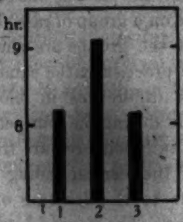

Fig. 3.

Fig. 2. Exp. 77, dog, 12 kg. Both splanchnic nerves cut. Ordinate: mean survival time of rats in hours. Number at the foot of each column indicates number of sample with which the group was treated. At each injection administration of either 0·3 ml. plasma (samples 1–3) or 0·5 ml. plasma (samples 4 and 5). Period of collection of samples 1–3, 4 min. *Sample* 1: control. Average blood pressure 133 mm. Hg. Blood flow 5·5 ml./min. *Sample* 2: during the last 4 min. of an infusion of 81 μg. adrenaline begun 2¼ min. previously. Average blood pressure 151 mm. Hg. Blood flow 5·5 ml./min. *Sample* 3: after 20 min. interval. Average blood pressure 103 mm. Hg. Blood flow 5·1 ml./min. *Sample* 4: mixture of two-fifths suprarenal plasma with three-fifths arterial plasma. *Sample* 5: same suprarenal plasma as that used in sample 4, without any arterial plasma.

Fig. 3. Exp. 79, dog, 11·4 kg. Both splanchnic nerves cut. Ordinate: mean survival time of rats in hours. Number at the foot of each column indicates number of sample with which the group was treated. 0·3 ml. plasma administered at each injection. Collection time for each sample 11 min. *Sample* 1: control period. Average blood pressure 53 mm. Hg. Blood flow 1·59 ml./min. *Sample* 2: 12 min. after the infusion of 80 μg. adrenaline in 6 min. Average blood pressure 40 mm. Hg. Blood flow 0·96 ml./min. *Sample* 3: 31 min. after sample 2. Average blood pressure 26 mm. Hg. Blood flow 1·02 ml./min. Saline is given intravenously before and during collection of sample 3.

and the blood flow through the gland 1·73 ml./min. Similar values are represented among the control samples, as shown above in Table 1.

The rise in yield of cortical hormone may start within a few minutes of the beginning of the adrenaline infusion. Column 2 of Fig. 2 (Exp. 77), for instance, represents the survival time obtained with a sample collected during the last 4 min. of an administration of adrenaline (81 μg., 6·7 μg./kg.) lasting 6·5 min. The sample contained a significantly larger amount of cortical hormone than the 4 min. sample collected before the infusion (column 1).

In Exp. 77, a further increase in cortical hormone was found, when a third 4 min. sample was taken 20 min. after cessation of infusion (column 3). No later sample was taken from this dog, but this was done in Exp. 79 (Fig. 3), in which a similar dosage of adrenaline (total of 80 μg. or 7 μg./kg. infused in 6 min.) had been used. Whereas the content of cortical hormone in a sample taken 12–23 min. after the end of the adrenaline infusion (column 2) was significantly greater than that of the control sample (column 1), it had dropped to the original level 54–65 min. after the infusion (column 3). Thus, the approximate duration of the action of adrenaline, in a dose of about 7 μg./kg. administered within 6 min., appears to be more than 20 min. and less than 1 hr. In the experiment illustrated in Fig. 1, the output of cortical hormone had returned to normal in sample 4, collected 17–34 min. after the end of infusion. The comparatively rapid disappearance of the effect in this instance was probably due to the slow infusion rate employed.

The amounts of adrenaline used in these three experiments are of the same order as those released into the circulation by electrical stimulation of the splanchnic nerves of one side (cf. Table 3, p. 327). We are, therefore, dealing with doses of adrenaline which may occur in the body under physiological conditions.

One experiment was done with a smaller dose, a total of 60 μg. (4·5 μg./kg.) being infused in 4 min. No change in the yield of cortical hormone resulted. Others were carried out with larger quantities, 3–4 times the doses employed in the experiments represented in Figs. 1–3. Two examples (Exps. 67 and 73) are given in Fig. 4. Column 1 indicates the yield in the control period, column 2 that during the infusion, and column 3 the result about half an hour later. The output rises considerably during the administration of the drug, and a further significant rise follows the infusion.

The periods of administration of adrenaline in Exp. 62 (Fig. 1) and Exp. 73 (Fig. 4 *b*) are nearly equal and the results are, therefore, comparable. The larger dose of adrenaline caused a more prolonged increase in the production of cortical hormone than the smaller one.

On further increasing the amount of adrenaline to quantities which are certainly no longer physiological, the result was still the same. The highest dose tested was one of 1512 μg. (226 μg./kg.) infused in 24 min. As may be seen from Fig. 5, the character of the response was unchanged.

The results described so far were obtained on dogs. An example of the same phenomenon in cats is seen in Fig. 6. The hormone content of the control sample 1 is considerably less than that of sample 2, obtained during the infusion of 77 μg. adrenaline (20·2 μg./kg.). The yield has not quite returned to normal in sample 3, collected 11–26 min. after the infusion had been discontinued. Column 4 measures the output of the gland in terms of the cortical extract 'Eucortone', the rats of group 4 having been injected with arterial plasma containing 6% eucortone. The comparison shows that, under the influence of the adrenaline infusion, the suprarenal plasma becomes slightly more potent than a 6% solution of this cortical extract.

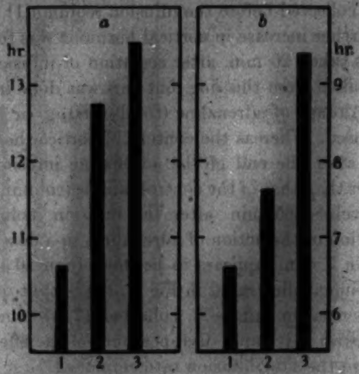

Fig. 4. Ordinates: mean survival time of rats in hours. Number at the foot of each column indicates number of sample with which the group was treated. 0·3 ml. plasma given at each injection.

(*a*) Exp. 67, dog, 9 kg. Both splanchnic nerves cut. Collection time of all samples 12 min. *Sample* 1: control period. Average blood pressure 117 mm. Hg. Blood flow 2·88 ml./min. *Sample* 2: during an infusion of a total of 204 μg. adrenaline started 2 min. previously. Average blood pressure 138 mm. Hg. Blood flow 2·71 ml./min. *Sample* 3: 26 min. after sample 2. Average blood pressure 64 mm. Hg. Blood flow 1·29 ml./min.

(*b*) Exp. 73, dog, 10 kg. Both splanchnic nerves cut. Collection time of all samples 16 min. *Sample* 1: control period. Average blood pressure 84 mm. Hg. Blood flow 1·89 ml./min. *Sample* 2: during an infusion of a total of 335 μg. adrenaline started 3 min. previously. Average blood pressure 98 mm. Hg. Blood flow 1·93 ml./min. *Sample* 3: 33 min. after sample 2. Average blood pressure 37 mm. Hg. Blood flow 0·78 ml./min.

Altogether, observations were made on twenty dogs and two cats. A significant increase in the output of cortical hormone was seen in thirteen of the twenty-two experiments; in one instance a rise did not occur *during*, but was seen in the sample collected 30 min. *after* the infusion. In six animals, no significant changes were observed. Considering the inaccuracy of the method, it is impossible to decide whether in these experiments the rise was absent or only smaller than usual; in one of them, the absence of effect was probably the result of using too small a dose of adrenaline.

In two experiments, adrenaline was given during a period of increased cortical secretion, caused in the one animal by a previous dose of adrenaline, and occurring spontaneously in the other for an unknown reason. Each time the hormone output dropped significantly from its raised level during the period of infusion. In the one dog in which a further sample was tested, the yield had again risen 11 min. later.

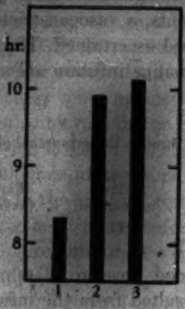

Fig. 5.

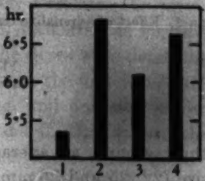

Fig. 6.

Fig. 5. Exp. 63, dog, 6·7 kg. Both splanchnics cut. Ordinate: mean survival time of rats in hours. Number at the foot of each column indicates number of sample with which the group was treated. 0·35 ml. plasma given at each injection. Collection time for each sample 19 min. *Sample* 1: control period. Average blood pressure 107 mm. Hg. Blood flow 1·62 ml./min. *Sample* 2: during an infusion of a total of 1·512 mg. adrenaline started 5 min. previously. Average blood pressure 134 mm. Hg. Blood flow 1·66 ml./min. *Sample* 3: 25 min. after sample 2. Average blood pressure 58 mm. Hg. Blood flow 0·90 ml./min.

Fig. 6. Exp. 51, cat, 3·8 kg. Both splanchnics cut. Ordinate: mean survival time of rats in hours. Number at the foot of each column indicates number of sample with which the group was treated. 0·35 ml. plasma administered at each injection. Period of collection 14·5 min. *Sample* 1: control period. Infusion of 40 ml. blood. Average blood pressure 39 mm. Hg. Blood flow 2·2 ml./min. *Sample* 2: obtained during infusion of a total of 77 μg. adrenaline begun 1½ min. previously. 12 ml. blood infused. Average blood pressure 82 mm. Hg. Blood flow 2·3 ml./min. *Sample* 3: after an interval of 11 min. 18 ml. blood infused just before, and 29 ml. during collection of sample. Average blood pressure 56 mm. Hg. Blood flow 2·1 ml./min. *Sample* 4: cat's arterial blood containing 6% 'Eucortone'.

In the experiments described in the first section of this paper, the minute output of cortical hormone was found to be independent of the blood flow through the gland. These observations were made under conditions in which cortical secretion was not increased; therefore, they do not exclude the possibility suggested by some of the foregoing observations that, provided the output of hormone is greatly enhanced, the blood supply to the suprarenals may be the limiting factor for the activity of the gland. For instance, the frequent observation that the full reaction to adrenaline does not develop before its administration is discontinued, may be attributed to a vasoconstriction present during the infusion. Similarly, restriction of blood flow might explain the temporary inhibition of cortical secretion encountered in the two experiments in which adrenaline was infused during a period of increased cortical activity. In many experiments, a vasoconstrictor effect of adrenaline on the suprarenal vessels was indeed ascertained. The changes in blood flow observed in the gland during adrenaline infusion are summarized in Table 2.

TABLE 2. Effect of adrenaline on the blood flow through the suprarenal glands

	Group 1 Flow decreased (average −8·5%)	Group 2 Flow unchanged	Group 3 Flow increased (average +27%)
No. of observations	4	11	9

In the three groups, an average rise in blood pressure of approximately the same size (22, 25 and 26 mm. Hg) had resulted from the infusion. (The rise was necessarily small, even with fairly large doses of adrenaline, since the animals were being bled during the infusion.) Vasoconstriction had undoubtedly taken place in the fifteen experiments of groups 1 and 2. It might also have been present in some or all experiments of group 3, and its effect on blood flow outweighed by the rise in blood pressure. It is interesting to note that Brauch, Brown & Rein [1935], using the thermostromuhr, regularly observed a diminution of suprarenal blood flow as a result of single intravenous injections of adrenaline into dogs anaesthetized with pernocton.

Since hypertrophy of the suprarenal cortex, whatever its causation, seems to be dependent upon the corticotrophic hormone of the anterior hypophysis [see Tepperman, Engel & Long, 1943], the possibility had to be considered, whether the effect of adrenaline on cortical activity was also mediated by the pituitary. This, however, was not the case, as proved by an experiment on a decapitated dog. In this animal, a dose of 60 μg. adrenaline (9·5 μg./kg. given as an infusion of 9 min. duration) caused a significant increase in output of cortical hormone.

Previous experience had shown [Vogt, 1943], that significant differences between mean survival times of groups of rats treated with solutions of cortical hormone are rarely seen, unless one sample contains about three times as much hormone as the other. We may, therefore, assume that the increase in hormone output caused by adrenaline, since it is usually found to be significant, represents a rise of several hundred per cent. It was only rarely possible to check this assumption by direct measurement, as the large number of rats required for that purpose was not often available. One experiment (77, Fig. 2) may serve as an example.

In addition to the three samples of blood (columns 1–3) required for the investigation of the effect of adrenaline, a fourth large sample of suprarenal blood was taken, and the plasma prepared from it ('plasma 4') was used for the evaluation of the differences obtained in the assay of the first three samples. For this purpose, 0·2 ml. of plasma 4 was given at each injection to one group, and a 2·5 times larger dose to another group of rats. The injected volumes were made equal by the addition of 0·3 ml. arterial plasma to the small dose of plasma 4. The survival times were 6·24 and 7·38 hr. (columns 4 and 5). On plotting these times against the logarithm of the doses, calling the small dose of 0·2 ml. unity, a line is obtained which correlates the survival times (ordinates) with doses of hormone (abscissae) measured as multiples of the small dose taken as unit. From this graph, the doses were determined which corresponded to the survival times observed with the first three samples, one sample having been taken before, one during, and one after adrenaline infusion. The figures obtained were 0·6, 1·8 and 5·0. This means, that during adrenaline infusion, the suprarenal blood contained three times as much hormone as in the control period, and that a further threefold rise occurred in the period following the infusion. On account of the low accuracy of the method, these results must be regarded as approximations.

Section and stimulation of the splanchnic nerves

Stimulation of the splanchnic nerves, by virtue of its release of adrenaline into the general circulation, is bound greatly to increase the activity of the suprarenal cortex. An attempt was made in the following experiments to decide whether there exist any additional effects of these nerves on the secretion of the suprarenal cortex.

Contradictory views have been held at different times on the relations between the splanchnics and the suprarenal cortex, but the most recent workers [Bennett, 1940; McFarland & Davenport, 1941] deny the existence of fibres innervating the cortex. According to these authors, the nerves penetrate the cortex without branching and make functional connexion with the medulla only. The example of the pituitary, however, where the *anterior* lobe can be stimulated by the hypothalamus, although nearly all fibres from the supraoptic nuclei end in the *posterior* lobe, illustrates that the absence of detectable nerve fibres is no proof of the absence of any nervous control. The close anatomical relationship between cortex and medulla, therefore, made it necessary to examine the possibility of some effect of the splanchnics on cortical activity being superimposed on their action through the release of adrenaline.

Splanchnotomy. In an anaesthetized animal with intact splanchnics, the operative manipulations preceding the collection of suprarenal blood inevitably cause adrenaline secretion. In order to test the effect of splanchnotomy on the suprarenal cortex without the interference of previous splanchnic stimulation, a period of rest of at least half an hour was allowed between the completion of the dissection and the collection of the first blood sample. When this sample had been obtained, the splanchnic nerves, under which ligatures had been placed in readiness during the operation, were rapidly divided. A second sample was then taken for assay. The result is illustrated in Figs. 7 and 8.

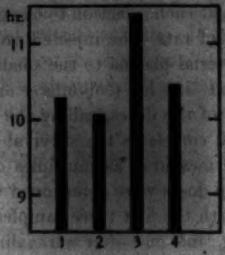

Fig. 7.

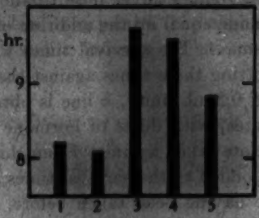

Fig. 8.

Fig. 7. Exp. 88, dog, 12 kg. Ordinate: mean survival time of rats in hours. Number at the foot of each column indicates number of sample with which the group was treated. 0·3 ml. given at each injection. Period of collection 6 min. *Sample* 1: obtained 30 min. after completion of all operative procedures. Average blood pressure 163 mm. Hg. Blood flow 4·6 ml./min. *Sample* 2: 6 min. after section of the splanchnic nerves on both sides. Average blood pressure 102 mm. Hg. Blood flow 2·3 ml./min. *Sample* 3: during stimulation of left splanchnic. Average blood pressure 86 mm. Hg. Blood flow 2·9 ml./min. *Sample* 4: after an interval of 1 min. Infusion of 25 ml. 0·9% NaCl. Average blood pressure 60 mm. Hg. Blood flow 2·0 ml./min.

Fig. 8. Exp. 91, dog, 11 kg. Ordinate: mean survival time of rats in hours. Number at the foot of each column indicates number of sample with which the group was treated. 0·4 ml. plasma given at each injection. Collection time of samples 12·5 min. *Sample* 1: obtained 34 min. after completion of the operation. Average blood pressure 137 mm. Hg. Blood flow 2·3 ml./min. *Sample* 2: 3 min. after section of the splanchnic nerves on both sides. Average blood pressure 98 mm. Hg. Blood flow 2·0 ml./min. *Sample* 3: during stimulation of the left splanchnic nerves. Blood pressure 88 mm. Hg. Blood flow 2·1 ml./min. *Sample* 4: after 9 min. interval and during infusion of 40 ml. saline. Average blood pressure 52 mm. Hg. Blood flow 2·0 ml./min. *Sample* 5: 41 min. after sample 3. 50 ml. saline infused before and 50 ml. during collection of sample. Average blood pressure 64 mm. Hg. Blood flow 2·3 ml./min.

In both experiments, group 1 was injected with plasma collected after the resting period, the splanchnic nerves being intact, and group 2 with a sample taken after section of the nerves. In spite of the large difference in blood pressure during the two periods of collection, the yield in cortical hormone is the same. Provided, therefore, stimuli have been excluded which give rise to the release of adrenaline from the suprarenal medulla, splanchnotomy does not affect the minute output of cortical hormone.

Stimulation of the splanchnic nerves. In order to guard against uncontrollable medullary activity due to reflex stimulation of the splanchnics, both before and during the collection of blood from the suprarenal vein, it was necessary to sever the nerves on both sides. The adrenaline released into the lumbar vein by the electrical stimulation cannot interfere, since it leaves the body with the venous effluent from the gland. Nevertheless, electrical stimulation of the left splanchnics produced an increase in the yield of cortical hormone in more than half of the experiments, whereas no change was observed in the remaining ones. Details are given in Table 3. In some instances, the increased production outlasted stimulation for some time, whereas in others recovery occurred immediately. In two animals (45 and 82), a rise in cortical activity was only seen after the stimulation. Examples of the time course are given in Figs. 7 and 8. In both figures, column 3 represents the yield during stimulation. It is significantly higher than that of the controls (column 2). In Fig. 7, column 4 shows the output immediately following stimulation: it has returned to the level of samples 1 and 2. In the other experiment (Fig. 8), collection of sample 4 was begun 9 min., and that of sample 5 41 min. after the period of stimulation. Since the collection lasted 12·5 min., the figure

TABLE 3. Effect of stimulation of the left splanchnic nerves on minute output of cortical hormone

No. of exp.	Period of stimulation min.	Adrenaline produced during stimulation: Total µg.	µg./kg.	Yield of cortical hormone during stimulation	Time course of yield after cessation of stimulation
37	10·5	93	6·8	Increased	Partial recovery 7 min. after stimulation
39	6·5	86	8·4	Increased	Partial recovery 11 min. after stimulation
48	7	63	2·5	Increased	Normal 20 min. after stimulation
52	30	216	19·0	Increased	—
88	6	36	3·0	Increased	Normal immediately after stimulation
89	7	58	5·8	Increased	Increased immediately after, normal 25 min. after stimulation
91	12·5	63	5·7	Increased	Increased 9 min. after, nearly normal 41 min. after stimulation
53	21	80	6·3	Probably increased	Normal immediately after stimulation
45	6·0	24	2·0	Unchanged	Increased 19 min. after stimulation
82	9	76	8·0	Unchanged	Increased 17 min. after stimulation
29	12	56	3·4	Unchanged	—
46	19	84	7·6	Unchanged	Normal 17 min. after stimulation
49	23·5	154	8·1	Unchanged	Normal 23 min. after stimulation
90	6	40	2·0	Unchanged	Normal 20 min. after stimulation
		Mean	6·3		

shows that the yield of hormone remained elevated between the ninth and twenty-first minute after stimulation, whereas another 20 min. later it was returning to resting level.

Atropine, which was given in Exps. 37 and 39 (Table 3), did not affect the result. In two dogs, however, in which the right splanchnics had not been severed, a reversible, significant fall in hormone production was observed during stimulation. It is possible that here reflex stimulation of the right splanchnic nerves during the operation had caused the release of sufficient adrenaline to maintain an elevated level of cortical activity during the collection of the 'control' sample, and that the decreased yield was analogous to that seen on two occasions (cf. p. 323) when adrenaline was administered during a period of increased cortical secretion. As this evanescent action of adrenaline had been attributed to vasoconstriction, information about the vasomotor effects of splanchnic stimulation is required before a similar explanation can be suggested for these experiments. Since in all experiments the dogs were eviscerated, splanchnotomized and were being bled from the lumbar vein during the stimulation of the splanchnics, the average change in blood pressure resulting from the stimulation was either a small rise only or occasionally even a slight fall. A steep fall in pressure, however, which regularly occurred when stimulation was discontinued, showed that the stimulus had been effective throughout the period of collection, but that its vascular effects were unable to compensate for the loss of blood incurred.

The changes in blood flow observed were not quite consistent. In eight experiments, the flow remained either unchanged or fell by 10–50% (mean 24%), despite a small rise in average blood pressure. Here vasoconstriction undoubtedly had taken place. In five experiments, a small increase in blood flow was seen to accompany a small rise in blood pressure: the resistance of the vascular bed had probably not changed. Three times the blood flow was augmented during stimulation in spite of a fall in the average blood pressure: vasodilatation had evidently occurred. Vasoconstriction of the suprarenal vessels, therefore, results frequently, but not invariably, from stimulation of the splanchnic nerves under the conditions of these experiments.

Arterial injection of acetylcholine

Injection of acetylcholine into the coeliac artery causes a release of adrenaline not only from the left suprarenal, the blood of which is drained away by the cannula, but also from the right gland which secretes into the animal. In order, therefore, to make the conditions comparable with those of the foregoing experiments on stimulation of the splanchnic nerves of the left side, the right suprarenal was excluded from the circulation by ligating its vascular connexions.

Three experiments were performed, particulars of which are given in Table 4. Their common feature was an increased output of cortical hormone *following* the administration of acetylcholine. *During* the injections, the yield remained unaffected in the first two, and was raised in the third animal, the only one in which the average blood pressure was not reduced during the injection period and the flow had greatly increased. This suggests that the delay in the onset of increased activity seen in the other two dogs had been caused by insufficient blood supply to the gland. In Exp. 82, a comparison was carried out on the same animal of the effect of splanchnic stimulation with that of acetylcholine injections. The results were indistinguishable. The output of cortical hormone was unaffected during, and significantly increased 17–26 min. after the electrical or chemical stimulation. Incidentally, the amounts of adrenaline released into the suprarenal effluent were also identical.

TABLE 4. Effect of intra-arterial injections of acetylcholine on minute output of cortical hormone

No. of exp.	Atropine given	Amount of acetylcholine injected	Period of injections min.	Response of blood pressure	Change in blood flow %	Adrenaline produced during injections		Yield of cortical hormone	
						Total µg.	µg./kg.	During the injections	After the injections
82	No	36 doses of 0·1 mg.	9	Fall of 32 mm. Hg	−30	76	8	Unchanged	Increased 17 min. later
84	Yes	32 doses of 0·1 mg.	11·5	Fall of 8 mm. Hg	+ 4	53	4·2	Unchanged	Increased immediately
86	Yes	26 doses of 0·1 mg.	8	No change	+45	150	15·8	Increased	Further increased immediately, normal 34 min. later

In the three experiments, the effect of the acetylcholine injections on the suprarenal blood vessels was a dilatation, whether or not atropine (4 mg.) had been given.

DISCUSSION

The foregoing experiments have demonstrated a hitherto unknown action of adrenaline. The hormone of the suprarenal medulla augments the continuous secretion of hormone by the suprarenal cortex. This action is obtained with amounts of adrenaline which are liable to be liberated in the body on splanchnic stimulation. Within a few minutes of the beginning of an adrenaline infusion, the output of cortical hormone rises to several times its resting value, and remains elevated or increases even further during a considerable period after cessation of the administration. This effect of adrenaline can be considered as a further and, probably, physiologically important item on the long list of effects by which adrenaline enables the organism to withstand sudden stress or strain.

Such an action of adrenaline explains certain observations on the correlation between cortical and medullary activity for which an interpretation was hitherto missing. Sjöstrand [1934], for instance, was struck by the fact that cortical hyperaemia in mice occurred in many conditions which are known to cause adrenaline secretion, and was led to the conclusion of the existence of a synergism between cortex and medulla. Looking through Sjöstrand's results, we find conspicuous hyperaemia recorded in animals subjected to (1) painful excitement, (2) asphyxia, (3) acetylcholine injection after eserine, (4) nicotine injection, (5) excitation at the beginning of anaesthesia, (6) distension of the intestine in anaesthesia. There is no doubt that these are conditions leading to adrenaline secretion, and, therefore, as we now know, to increased cortical output. The correlation observed by Sjöstrand is thus easily explained, as a larger blood flow will be required by the more rapidly secreting cortex.

In 1912, Neumann discovered that an injection of adrenaline causes a considerable rise in the oxygen consumption of the suprarenal gland. A corresponding observation was made by Broening [1924], who stimulated the splanchnic nerves, and recorded an increase in suprarenal blood flow of about 50% and a rise in oxygen consumption of about 200–300%. Although, at the period of those observations, the interest centred on the secretion by the medulla, there is hardly any doubt that the large increase in metabolism resulting from adrenaline injection or splanchnic stimulation is shared by the cortex. In the case of an adrenaline injection, it may even be entirely accounted for by the enhanced cortical activity. This increase in oxygen consumption provides an explanation for the few apparently aberrant results, in which a fall in cortical hormone production resulted from administration of adrenaline or from splanchnic stimulation. Such a diminished output was only observed when adrenaline was infused during a period of increased cortical activity, or when stimulation was applied under conditions where one could assume that reflex stimulation of the splanchnic nerves had occurred shortly before the sample was taken. The suprarenal vasoconstriction, which has been shown to accompany, as a rule, both infusion of adrenaline and stimulation of the splanchnics, may curtail the oxygen supply sufficiently to decrease temporarily an elevated yield of cortical hormone. The rapid recovery from the depression is in good agreement with this interpretation. Similarly, the fact that not infrequently the increase in cortical output did not occur, or reach its full height, till after the cessation of infusion or stimulation, may be due to limitation of the oxygen supply by the vascular effects of these procedures.

The stimulation of cortical activity by adrenaline is a direct effect on the secretory cells, independent of the anterior lobe of the pituitary. It is one of the rare instances in which adrenaline acts upon a structure devoid, as far as we know, of a sympathetic nerve supply. In its duration, which greatly exceeds that of the effects of adrenaline on smooth muscle, this action resembles that on transmission of nervous impulses, although it differs from it in the absence of any inhibitory effects by large amounts [Bülbring & Burn, 1942; Bülbring, 1944].

Through the action of circulating adrenaline, the sympathetic system exerts an indirect nervous control over the suprarenal cortex. This takes the place of the direct innervation in which the cortical tissue is apparently lacking. It is not easy, however, to interpret the experiments in which splanchnic stimulation also augmented cortical output, when the adrenaline secreted into the lumbar vein was prevented from entering the circulation. Either the activity of the nerve endings in the medulla may affect the cortex by some unknown mechanism, in a fashion which simulates the action of adrenaline, or some adrenaline may reach the cortical cells, even in a preparation in which the blood from the lumbar vein is being drained off. The second alternative appears the more likely, considering that this effect of splanchnic stimulation is not obtained regularly, and that section of the splanchnic nerves does not alter the output of cortical hormone; but neither experimental nor anatomical data provide conclusive information. Although the structure of the vascular tree of the suprarenal gland [Flint, 1900; Bennett & Kilham, 1940] does not favour the view that medullary blood containing adrenaline is able to reach the cortex, some degree of back diffusion from the medullary veins cannot be ruled out with certainty. It is, further, open to question whether or not some adrenaline might by-pass the lumbar vein, and reach the general circulation and from there the cortex. One path might be small veins leaving the suprarenal capsule and accompanying the small arteries which enter the gland. They cannot be tied in the preparation without endangering the arterial supply. Though they mainly contain blood which has been irrigating the cortex, some leakage of adrenaline into these channels remains possible. A more important source of adrenaline which cannot be easily checked is, however, provided by the aortic paraganglia which do not become atrophic in the adult dog [Iwanow, 1932]. The physiology of these bodies is rather obscure, but their intimate contact with sympathetic nerves by which they appear to be innervated [Smirnow, 1890; Kohn, 1903], and their content of extractable adrenaline [Biedl & Wiesel, 1902] are known. It is, therefore, not unlikely that stimulation of the splanchnic nerves will cause a release of adrenaline from this chromaffine tissue which may thus contribute appreciably to the adrenaline level in the blood.

The close parallelism observed between the results of electrical stimulation of the splanchnics and of intra-arterial injection of acetylcholine indicates that the drug is devoid of any action on the suprarenal cortex which is not due to its excitation of structures innervated by cholinergic nerves.

SUMMARY

1. Intravenous infusion of adrenaline causes a strong, immediate and long-lasting stimulation of suprarenal cortical activity in the eviscerated dog or cat. The effect was obtained with doses of adrenaline which occur in the body under physiological conditions. The increased yield was of the order of several times the basal output. The action is independent of blood pressure or blood flow, and is not mediated by a hormone from the pituitary. Through adrenaline, the sympathetic nervous system has an indirect control over the activity of the suprarenal cortex.

2. Electrical stimulation of the left splanchnics, provided reflex activity of the nerves on both sides had been avoided, caused an increase in cortical secretion in half of the experiments, despite the fact that the adrenaline released into the lumbar vein was prevented from reaching the general circulation. This stimulation of the suprarenal cortex resembled in its time course that of a small dose of adrenaline. It is interpreted as probably due to some adrenaline having entered the general circulation through small accessory suprarenal veins, or having been produced by the aortic paraganglia.

3. Section of the splanchnics, if carried out under conditions where the nerves had not recently been stimulated, and the after-effects of circulating adrenaline could therefore be excluded, did not alter the 'basal' output of the suprarenal cortex.

4. Close arterial injection of acetylcholine into one suprarenal (the other being excluded from the circulation) was indistinguishable in its effect from that of stimulation of the splanchnic nerves of one side.

5. The effect of adrenaline infusion on the suprarenal vessels was usually a constriction, that of splanchnic stimulation frequently a constriction, whereas that of acetylcholine was a dilatation. There is some evidence that the vasoconstriction has occasionally a transient antagonistic effect on the increased cortical activity caused by adrenaline.

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THE INHIBITION OF HISTAMINE RELEASE BY A PITUITARY-ADRENAL MECHANISM

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There is experimental evidence that post-traumatic mortality is reduced in guinea-pigs and rats which have undergone a previous sublethal trauma [Noble, 1943; Ungar, 1943]. It has been shown, moreover, that this resistance can be passively transferred to intact animals by the injection of the serum of traumatized animals [Ungar, 1943]. The present paper is mainly concerned with the explanation of this phenomenon while other processes of acquired resistance to 'shock' are also considered.

METHODS

The technique used throughout the experiments is based on the observation published by Gotzl & Dragstedt [1942] that blood of normal rabbits, when mixed with peptone, releases *in vitro* considerable amounts of a substance pharmacologically identifiable as histamine. This fact was confirmed and extended by using the following procedure:

Blood was collected by heart puncture from rabbits and guinea-pigs, and by section of the neck from rats; 0·5 c.c. was mixed with 1 c.c. of 0·9% solution of NaCl (*A*), and 0·5 c.c. with 1 c.c. of a 1·3% solution of peptone in saline (*B*). Coagulation was prevented with heparin or 'Liquoid Roche'. After centrifuging for 10 min., the supernatant fluid was collected and diluted to 5 c.c.

Histamine was estimated by the usual technique, guinea-pig ileum serving as test object. Unless otherwise stated, results are given in μg. of histamine dihydrochloride per c.c. of blood. The amount of histamine released is given by the difference between the two samples *A* and *B*, taking into account the direct action of peptone (*P*) on the intestine ($H=B-A-P$).

The plasma was tested without previous extraction of the histamine-like substance. This was justified by comparing the results given by six samples tested both directly and also after extraction with Code's method [1937]. The mean value of the histamine released was 0·22 ± 0·08 μg./c.c. by direct estimation and 0·20 ± 0·06 with Code's technique. In a few cases the fragment of ileum was unsuitable either because it reacted strongly to some unknown plasma constituent or because it was inhibited by peptone.

The right amount of peptone to be used, the most convenient duration for the contact between peptone and blood and the effects of temperature were investigated. As peptone is far from being a well-defined chemical product, the dose may vary from one commercial preparation to another. Duration of contact, within the limits of 10 min. to 2 hr., does not influence the results. Neither was any difference observed between mixtures kept at 18 and 38° C.

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Trauma was administered by the quantitative method described previously [Ungar, 1943] in which the amount of trauma is expressed in kg.m. of energy spent by a metal rod of known weight falling on to the thigh of the animal from a measured height.

Results were tabulated as means of several (generally eight) identical experiments. Variation of the mean was expressed in terms of the standard error (S.E.). Tests for significance of the differences were carried out by means of the t test [Fisher & Yates, 1943]. In the tables, S beside a figure indicates that there is less than 1/20 chance that this figure represents the control amount of histamine released.

RESULTS

Histamine release in normal animals

It was confirmed that peptone releases histamine from rabbit blood *in vitro*. The average amount released in ten rabbits was 0·58 ± 0·09 μg./c.c. The amount determined by Gotzl & Dragstedt on the basis of experiments on five rabbits was 0·67 ± 0·12 μg./c.c. (Both these results are given in terms of histamine base.) The agreement is fairly good when allowance is made for the fact that in one case histamine estimation was done directly and in the other after extraction.

In guinea-pig blood the histamine release, although less pronounced, is still measurable and is quantitatively fairly constant. In thirty-nine adult guinea-pigs (20 ♂ and 19 ♀) the mean release was 0·19 ± 0·02 μg./c.c. For males the mean release was 0·18 ± 0·02, and for females 0·20 ± 0·03 μg./c.c. Ten immature guinea-pigs (each weighing less than 300 g.) gave a lower figure, 0·13 ± 0·015 μg./c.c. Although the difference between the adult and immature specimens is not significant, all subsequent experiments were performed on adult animals.

In the same conditions rat blood also releases histamine. In fourteen adult rats of both sexes the mean release was 0·15 ± 0·02 μg./c.c.

Since the amount of histamine released in the blood of normal animals shows comparatively little variation, the above figures can legitimately be used for the control of experimental data.

Histamine release after injection of peptone

The object of the first series of experiments was the study of histamine release in the blood of animals which had received an injection of peptone and were therefore in a refractory state. Table 1 shows that blood taken from

TABLE 1. The effect of injections of peptone on the histamine release

Dose of peptone	Interval	Histamine μg./c.c.	±S.E.	No. of animals	Significance
None. Control	—	0·19	0·02	39	
500 mg./kg.	2½ hr.	0·04	0·02	8	S
„	24 hr.	0·04	0·03	7	S
„	48 hr.	0·03	0·015	7	S
100 mg./kg.	1 hr.	0·06	0·025	8	S
„	20 hr.	0·11	0·02	8	S
„	48 hr.	0·18	0·04	8	
50 mg./kg.	2 hr.	0·12	0·05	8	
10 mg./kg.	2 hr.	0·24	0·02	8	
„	24 hr.	0·17	0·05	8	

guinea-pigs which have been given a subcutaneous injection of more than 50 mg./kg. of peptone released significantly less histamine than normal guinea-pig blood. The duration of this reduction of histamine release varies with the dose of peptone.

It may be assumed that the decrease of histamine release *in vitro* is the expression of the refractory phase which is known to follow the injection of peptone.

The effect of trauma

In order to investigate whether the resistance to trauma induced by a previous injury is a condition related to peptone refractoriness, blood of traumatized guinea-pigs was examined. Varying amounts of trauma were administered under ether anaesthesia and blood samples collected at different intervals.

TABLE 2. The effect of trauma on the histamine release

Amount of trauma	Interval	Histamine µg./c.c.	±S.E.	No. of animals	Significance
None. Control	—	0·19	0·02	39	
0·95 kg.m.	1 hr.	0·22	0·045	8	
„	2 hr.	0·275	0·05	8	
„	3 hr.	0·225	0·03	8	
„	4 hr.	0·05	0·02	15	S
„	24 hr.	0·1	0·035	7	S
„	48 hr.	0·04	0·02	8	S
„	3 days	0·04	0·03	7	S
„	5 days	0·06	0·03	8	S
„	7 days	0·04	0·01	8	S
„	9–10 days	0·11	0·03	15	S
„	14–15 days	0·09	0·02	12	S
„	19 days	0·1	0·025	10	S
„	23–24 days	0·22	0·03	9	
„	29 days	0·27	0·04	6	
0·42 kg.m.	3 days	0·075	0·03	8	S
„	8 days	0·11	0·03	7	S
„	11 days	0·27	0·08	6	
0·21 kg.m.	5 hr.	0·05	0·02	8	S
„	48 hr.	0·08	0·03	7	S
„	4 days	0·11	0·015	7	S
„	6 days	0·17	0·01	7	
„	8 days	0·21	0·05	7	
0·055 kg.m.	5 hr.	0·25	0·03	8	
„	24 hr.	0·27	0·025	8	
None. Ether anaesthesia	—	0·05	0·01	6	S
„	1 hr.	0·16	0·015	6	
None. Haemorrhage, 1% body weight	4 hr.	0·24	0·01	7	
„	24 hr.	0·24	0·015	8	

Results, shown in Table 2, clearly indicate that 4 hr. after trauma histamine release *in vitro* is reduced and that the reduction lasts for a period related to the amount of trauma. With a trauma of 0·95 kg.m. the return to normal requires 19–23 days, with 0·42 kg.m. 8–11 days and with 0·21 kg.m. 4–6 days. The recovery of the normal histamine release coincides with the clinical healing of the wound. A control series of animals, submitted to ether anaesthesia but not traumatized, shows that while blood of animals under ether releases little histamine, the amount released is again normal 1 hr. later. The purpose of another control series of experiments was the study of the effect of direct blood loss. Haemorrhage amounting to 1% body weight has no effect.

Passive transfer of resistance

Table 2 shows that increased resistance to trauma, like peptone refractoriness, is accompanied by an inhibition of the histamine release *in vitro*. The next step was to examine whether this inhibition can be induced by the injection of the serum of resistant animals. In these experiments blood samples were collected 2 hr. after subcutaneous injection of sera taken, at varying intervals, from guinea-pigs which had been traumatized with 0·95 kg.m. Results of these experiments were compared with those given by the serum of animals treated with peptone or having had an anaphylactic shock. The latter guinea-pigs had been sensitized with egg albumin and 3 weeks later were tested intracardially with the antigen. All showed signs of shock but recovered and were subsequently killed and bled.

TABLE 3. Passive transfer of the inhibition of histamine release

Serum from	Dose c.c./kg.	Histamine μg./c.c.	±S.E.	No. of animals	Significance
No injection. Control	—	0·19	0·02	39	
Normal guinea-pigs	2	0·175	0·025	12	
Guinea-pigs traumatized under ether 6 hr. previously	0·001	0·06	0·02	8	S
”	0·0001	0·17	0·02	8	
Guinea-pigs traumatized under ether 24 hr. previously	0·001	0·06	0·02	8	S
”	0·0001	0·21	0·03	8	
Guinea-pigs traumatized under urethane 6 hr. previously	2	0·15	0·035	4	
Guinea-pigs traumatized under urethane 24 hr. previously	0·01	0·03	0·01	8	S
”	0·001	0·19	0·04	8	
Guinea-pigs injected with 500 mg./kg. peptone 4 hr. previously	0·1	0	—	8	S
”	0·01	0·22	0·04	8	
Guinea-pigs injected with 500 mg./kg. peptone 24 hr. previously	2	0·025	0·01	8	S
”	0·1	0·125	0·025	8	
”	0·01	0·175	0·02	8	
Guinea-pigs having had anaphylactic shock 3 hr. previously	0·5	0·06	0·02	8	S
”	0·1	0·19	0·035	8	
Guinea-pigs having had anaphylactic shock 24 hr. previously	0·5	0·01	0·005	8	S
”	0·1	0·18	0·03	8	

Table 3 shows the limit of activity of sera collected from resistant animals. Guinea-pigs injected with these sera release significantly less histamine than normal animals or animals injected with normal guinea-pig serum. The activity of various sera shows considerable variation according to the conditions in which resistance has been acquired. For clearer understanding of the results, the activity of sera may be expressed in terms of a unit defined as the minimum dose which, when injected into normal guinea-pigs, significantly reduces the amount of histamine liberated *in vitro*. In this way, serum from normal guinea-pigs contains less than 0·5 unit/c.c., while serum from animals traumatized under ether contains between 1000 and 10,000 units/c.c. This difference may explain why peptone refractoriness cannot be passively transferred, whereas resistance to trauma can. Experience has shown that, in order to produce effective protection against peptone shock or trauma, several hundred units must be injected per kg. body weight.

Table 3 also shows that urethane tends to prevent the development of resistance. Serum of guinea-pigs traumatized under urethane is not more active than normal serum and activity develops only when the anaesthetic effect of urethane comes to an end (between 100 and 1000 units/c.c. 24 hr. after). These findings agree with other experimental facts. Subcutaneous injection of 500 mg./kg. of peptone into eight guinea-pigs under urethane anaesthesia (1 g./kg.) resulted in 100% mortality, whereas the same treatment given to unanaesthetized guinea-pigs failed to kill any. It was also shown in a previous paper [Ungar, 1943] that trauma of 3·8 kg.m. administered in specified conditions to urethanized guinea-pigs resulted in 100% mortality. The same amount of trauma given in the same conditions but under ether anaesthesia had no lethal effects.

Anaphylactic shock produces a serum of low activity. It seems certain that anaphylactic desensitization is brought about by means of a saturation of antibodies which cannot be transferred. The slight activity of sera (between 2 and 10 units/c.c.) may perhaps explain the relative protection afforded by anaphylactic shock against the effect of peptone.

The action of drugs

The next question examined was whether histamine release can be affected by drugs. All drugs were given subcutaneously, with the exception of urethane which was injected intraperitoneally.

Table 4 shows that histamine, urethane, adenosine phosphoric acid and aneurine have no effect on histamine release. Among the substances which inhibit histamine release are those that showed a definite beneficial action on post-traumatic mortality, e.g. ascorbic acid and nupercaine [Ungar, 1943]. The better results in preventing post-traumatic mortality obtained with nupercaine can now be explained by its quick and long lasting action. On the other hand, the long delay between the administration and the beginning of the action of ascorbic acid may explain certain reports of its failure to

TABLE 4. Action of drugs on the histamine release

Drug	Dose per kg.	Interval	Histamine μg./c.c.	±S.E.	No. of animals	Significance
Control	—	—	0·19	0·02	39	
Histamine HCl	2·5 mg.	3 hr.	0·165	0·03	7	
Urethane	1 g.	1 hr.	0·22	0·04	20	
"	1 g.	24 hr.	0·19	0·05	7	
Adenosine phosphoric acid	10 mg.	3 hr.	0·19	0·02	8	
"	10 mg.	24 hr.	0·24	0·03	7	
Aneurine	10 mg.	3 hr.	0·28	0·05	8	
Ascorbic acid	100 mg.	½ hr.	0·2	0·025	8	
"	100 mg.	1 hr.	0·16	0·04	8	
"	100 mg.	2 hr.	0·08	0·03	8	S
"	100 mg.	3 hr.	0·04	0·02	8	S
"	100 mg.	6 hr.	0·04	0·02	8	S
"	100 mg.	13 hr.	0·06	0·03	8	S
"	100 mg.	24 hr.	0·16	0·03	8	
"	100 mg.	48 hr.	0·19	0·03	7	
Nupercaine HCl	4 mg.	12 min.	0·09	0·025	8	S
"	4 mg.	½ hr.	0·04	0·02	8	S
"	4 mg.	4½ hr.	0·1	0·03	8	S
"	4 mg.	24 hr.	0·06	0·02	7	S
"	4 mg.	48 hr.	0·2	0·01	7	
Procaine HCl	100 mg.	4½ hr.	0·07	0·02	8	S
"	100 mg.	24 hr.	0·19	0·045	8	
"	100 mg.	48 hr.	0·185	0·025	7	
Cocaine HCl	40 mg.	3 hr.	0·06	0·02	8	S
"	40 mg.	24 hr.	0·07	0·03	8	S
Adrenaline HCl	0·1 mg.	2 hr.	0·03	0·01	8	S

protect against anaphylactic and peptone shock [Eyer, Dragstedt & Ramirez, 1938; Dragstedt, Eyer & Ramirez, 1938]. Table 4 also shows that procaine has a comparatively short action on histamine release; this may explain its inability to prevent post-traumatic mortality. As cocaine was also found active, it is possible that the power to inhibit histamine release *in vitro* is in some way related to local anaesthetic action. High, unphysiological doses of adrenaline also have an effect on histamine release.

It could be asked whether the inhibition of histamine release induced by drugs can be transferred to non-treated guinea-pigs by injecting the serum of treated animals. Serum of animals treated with ascorbic acid or nupercaine was therefore injected into normal guinea-pigs. Blood from eight guinea-pigs injected with 1 c.c./kg. of ascorbic acid serum released 0·19 ± 0·02 μg./c.c. histamine. Nupercaine serum injected into eight guinea-pigs at a dose of 0·1 c.c./kg. gave a release of 0·07 ± 0·02 μg./c.c. and at a dose of 0·01 c.c./kg. gave 0·21 ± 0·03 μg./c.c. It seems therefore probable that ascorbic acid has a direct action since the serum of animals treated with this substance is inactive. Nupercaine, on the other hand, probably acts by producing some other substance which is contained in the serum of treated animals in amounts of 10–100 units/c.c.

The effect of adrenalectomy

The following series of experiments was designed to determine the origin of the inhibitory substance present in the serum. An attempt was first made to determine the part played by the adrenals in the production of this substance. Table 5 shows a comparison between normal rats and rats adrenalectomized 2 days previously and maintained on salt water. It is clear that normal rats, like guinea-pigs, release less histamine when subjected to peptone, trauma, ascorbic acid, nupercaine, adrenaline, adrenal cortical extract and serum of traumatized animals. Adrenalectomized rats, however, are affected only by ascorbic acid and cortical extract. The action of ascorbic acid was to be expected from the results of experiments with the serum of guinea-pigs treated with this substance. A crude cortical extract at a dose corresponding to 0·1 mg. of fresh gland inhibited the histamine release both in normal and adrenalectomized animals. However, the active substance of this extract cannot be the same as the inhibitory substance of the serum since the latter has no action in adrenalectomized animals.

TABLE 5. Histamine release in the blood of normal and adrenalectomized rats

	Normal rats				Adrenalectomized rats			
Treatment	Histamine μg./c.c.	±S.E.	No. of animals	Significance	Histamine μg./c.c.	±S.E.	No. of animals	Significance
None. Control	0·15	0·02	14		0·27	0·07	12	
Peptone, 500 mg./kg.	0·03	0·01	8	S	0·16	0·02	8	
Trauma, 1·9 kg.m.	0·04	0·02	8	S	0·22	0·03	7	
Ascorbic acid, 100 mg./kg.	0·025	0·02	8	S	0·01	0·01	7	S
Nupercaine, 20 mg./kg.	0·03	0·01	8	S	0·21	0·035	8	
Adrenaline, 0·5 mg./kg.	0·03	0·01	8	S	0·21	0·02	8	
Adrenal cortical extract, 0·1 mg./kg.	0·025	0·01	8	S	0·03	0·02	8	S
Serum of traumatized guinea-pigs, 2 c.c./kg.	0·03	0·01	8	S	0·29	0·06	8	

The effect of adrenalectomy and hypophysectomy on the production of the inhibitory substance

While the adrenals seemed to be indispensable for the inhibition of histamine release (except in the case of ascorbic acid), the site of production of the inhibitory substance of the serum was to be found elsewhere. An obvious organ to be examined was the pituitary body. Tepperman, Engel & Long [1943], reviewing the literature of adrenal cortical hypertrophy, concluded that changes in the adrenals can only be brought about through the pituitary. In order to examine the parts played by the adrenals and pituitary in the production of the inhibitory substance, guinea-pigs were injected with serum collected from normal, adrenalectomized and hypophysectomized rats submitted 5 hr. previously to a trauma of 0·95 kg.m. Guinea-pigs injected with normal rat serum were used as controls.

Table 6 shows that serum of intact traumatized rats has an activity of the same order as that of similarly treated guinea-pigs (1000–10,000 units/c.c.). The serum of adrenalectomized and traumatized rats has the same activity.

TABLE 6. Activity of sera from normal, adrenalectomized and hypophysectomized rats after trauma

Serum from	Dose c.c./kg.	Histamine μg./c.c.	±S.E.	No. of animals	Significance
None. Control	—	0·19	0·02	39	
Normal rats	0·1	0·22	0·02	8	
Normal traumatized rats	0·001	0·03	0·01	8	S
,,	0·0001	0·16	0·02	8	
Adrenalectomized traumatized rats	0·001	0·02	0·01	8	S
,,	0·0001	0·21	0·02	8	
Hypophysectomized traumatized rats	0·1	0·2	0·025	8	

Now, according to Table 5, blood from adrenalectomized and traumatized rats releases a normal amount of histamine. This can be explained by assuming that adrenalectomy does not prevent the production of the inhibitory substance but that this substance can only act through the adrenals. On the other hand, Table 6 indicates that the inhibitory substance is not produced in the absence of the pituitary.

Effect of adrenal and pituitary extracts

Adrenal and pituitary extracts were finally tested in guinea-pigs. The results summarized in Table 7 show that both glands contain highly active products. A crude adrenal extract in saline contains 100,000–1,000,000 units/g. of fresh tissue. A crude alkaline pituitary extract has the same activity. Liver extract, tested as control, contained no active principle in 100 mg.

TABLE 7. Effect of adrenal and pituitary extracts on histamine release

Extract	Dose* per kg.	Histamine μg./c.c.	±S.E.	No. of animals	Significance
None. Control	—	0·19	0·02	39	
Guinea-pig liver	100 mg.	0·17	0·02	8	
Rat adrenal	100 mg.	0·03	0·01	8	S
Guinea-pig adrenal	1 mg.	0·02	0·005	8	S
,,	0·01 mg.	0·03	0·01	8	S
,,	0·001 mg.	0·21	0·03	8	
Whole cortical extract, Kendall	1 mg.	0·025	0·02	8	S
,,	0·1 mg.	0·19	0·05	8	
Desoxycorticosterone acetate†	2 mg.	0·19	0·02	8	
,,	10 mg.	0·19	0·035	8	
Guinea-pig pituitary	0·01 mg.	0·05	0·02	8	S
,,	0·001 mg.	0·19	0·02	8	
'Antex'†	0·1 mg.	0·04	0·01	8	S
,,	0·01 mg.	0·21	0·02	8	
'Corticotrophin'†	0·01 μg.	0·04	0·02	8	S
,,	0·001 μg.	0·23	0·03	8	

* Unless otherwise stated, doses are given in weights corresponding to fresh tissue.
† Dose given in weight of pure product.

Purified products are less active. A whole cortical extract prepared by Kendall contains between 1000 and 10,000 units/g. of fresh tissue. Synthetic desoxycorticosterone acetate is inactive. A commercial preparation of anterior pituitary ('Antex') contained only between 10,000 and 100,000 units/g. of dry substance. However, the most powerful product tested so far is a corticotrophic hormone* containing between 10^8 and 10^9 units/g. of dry substance.

The most likely explanation of the inhibition of the normal histamine release in blood *in vitro* is that after certain stimuli (tissue damage, peptone injection, administration of drugs, etc.) the pituitary releases into the circulation an active principle that stimulates the adrenals, which in their turn act directly or indirectly on the blood cells. Further investigation is necessary in order to determine the mechanism of this action more precisely.

DISCUSSION

Liberation of histamine from isolated tissue fragments has already been used as a test for anaphylactic sensitivity and desensitization [Ungar & Parrot, 1936; Schild, 1937; Campbell & Nicoll, 1940]. Katz [1940] was the first to use blood for *in vitro* anaphylaxis. Histamine release can also be used as a test for peptone shock since Feldberg & O'Connor [1937], Dragstedt & Mead [1937] and Tinel, Ungar & Parrot [1938] have shown that peptone acts largely by releasing histamine from certain tissues. It is probable that lung tissue provides the main source of histamine in guinea-pig anaphylactic and peptone shock and that blood cells are only of secondary importance.

The fact that histamine liberation is used as a test does not imply that it is the only or even the main substance released. The reason for using histamine is that it can be detected in extremely small amounts by a comparatively easy technique.

Desensitization, which follows anaphylactic shock, is generally ascribed to the saturation of antibodies. No such explanation can be put forward to explain the refractory phase brought about by peptone. Peptone has no antigenic properties [Fink, 1919] and peptone shock therefore is not the expression of an antigen-antibody reaction. Mead, Dragstedt & Eyer [1937] and Dragstedt [1943] suggest that refractoriness is the result of the exhaustion of the histamine supply of certain cells and can therefore be brought about only after heavy shock and not at all by injection of small doses or by subcutaneous administration. If inhibition of the histamine release *in vitro* can be accepted as a criterion of refractoriness, this condition can without doubt be induced by small subcutaneous doses giving no shock and presumably no appreciable liberation of histamine. Moreover, Feldberg & O'Connor [1937] have found that in isolated and perfused lung a second injection of peptone may give rise to a higher histamine output than the first. Exhaustion of the histamine supply can play some part in anaphylactic desensitization as in peptone refractoriness but just as the primary cause in the first case is the saturation of antibodies, in the second it is probably some other process.

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Peptone refractoriness probably belongs to the non-specific conditions of resistance described under the name of tachyphylaxis [Champy & Gley, 1911] or skeptophylaxis [Ancel, Bouin & Lambert, 1911]. It is probably related to the phenomena described by Selye [1937] as adaptation to 'alarming' stimuli. The experiments reported in this paper may supply an explanation for the mechanisms involved in these reactions. Selye has shown that 'adaptation' is accompanied by an increase in the volume of the adrenal cortex. Tepperman *et al.* [1943] have reviewed the various conditions in which adrenal cortical hypertrophy had been observed, e.g. injection of peptone [Whitehead, 1932], exposure to excessive cold or heat, trauma, burns, starvation, muscular exercise, adrenaline, formaldehyde, etc., and they suggested that the common factor present in most cases to which the adrenals react is the presence of protein breakdown products in the circulating blood.

The experiments described in the present paper give confirmation of the part played by the adrenals and the pituitary in the non-specific protective or 'adaptative' process suggested by morphological observation. These experiments may also explain why adrenalectomized animals are highly sensitive to trauma [Freed, 1932; Swingle & Parkins, 1935; Swingle, Parkins, Taylor & Hays, 1938; Hechter, Krohn & Harris, 1942; Noble & Collip, 1942]. Increased sensitivity to various stimuli was also observed in hypophysectomized animals by Tyslowitz & Astwood [1942], Noble & Collip [1942], Joseph, Schweitzer & Gaunt [1943] and Reiss, Macleod & Golla [1943]. The latter writers have also observed that corticotrophic hormone protects animals against shock-like conditions.

The experiments described above suggest that certain stimuli bring about the secretion by the pituitary of a hormone included in the corticotrophic fraction. This hormone, through the mediation of the adrenal cortex, determines a change in the blood cells and probably other tissues which can be detected by the reduced capacity of blood cells to liberate histamine under the action of peptone. From the practical point of view the cellular change determined by the joint action of the pituitary and the adrenal cortex is beneficial to the animals which become resistant to any further action of the stimuli. The protection thus created is not specific, i.e. the refractory state created by peptone affords protection against the effects of tissue damage and *vice versa*. With certain quantitative limitations, the protective action can be transferred by injecting sera containing the active principle into animals not subjected to the original stimulus.

SUMMARY

1. Blood of normal rabbits, guinea-pigs and rats, when mixed *in vitro* with peptone, liberates histamine into the plasma. The amount of histamine released per c.c. of blood shows little variation within the same species.

2. Histamine release is significantly reduced in the blood of guinea-pigs and rats submitted to the action of peptone, trauma or certain drugs (adrenaline, local anaesthetics, ascorbic acid).

3. Inhibition of histamine release can be transmitted to normal animals by injection of the serum of traumatized animals. A unit is defined for measuring the activity of these sera.

4. Evidence is given that the inhibitory substance present in active sera is produced by the pituitary and acts through the adrenals.

5. The significance of the reaction is discussed from the point of view of peptone refractoriness, non-specific resistance and induced insensitivity to traumatic shock.

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AN OPTICAL METHOD FOR RECORDING PERIPHERAL BLOOD PRESSURES AND PULSE RATES IN UNANAESTHETIZED AND IN ANAESTHETIZED RABBITS

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(*Received* 10 *May* 1944)

A method of recording the systolic pressure in the ear artery of a rabbit is reported in this paper. It is an extension of the method described by Grant & Rothschild [1934], the arterial pulses being recorded on bromide paper. The records are therefore permanent and the subjective element in the measurements is avoided; also, the pulse rate can be counted on the records. Measurements may be made every quarter minute in anaesthetized and in unanaesthetized rabbits. The system includes a Grant ear capsule filled with water. At the end of the paper we describe the manufacture of a waterproof inelastic membrane for the capsule.

Description of apparatus

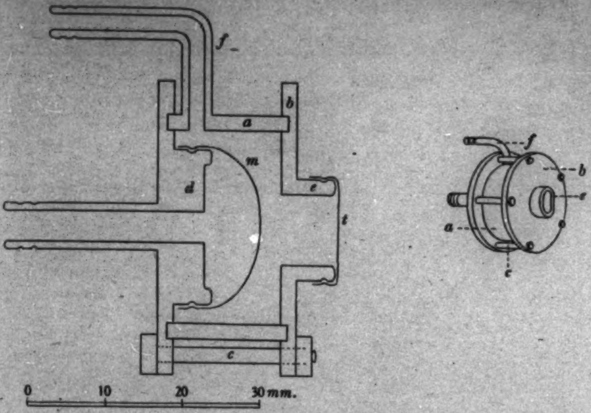
The method is based upon the obliteration of the pulse in a short length of the ear artery by pressure transmitted through the membrane of the Grant capsule. The pressure in the capsule is first raised high enough to obliterate the artery; then, as the pressure falls, a point is reached when blood spurts across the compressed section of the artery with each pulse: this point corresponds to the systolic pressure. The high occluding pressure is borne by a thick rubber membrane and the arterial pulses are picked up by a thin rubber membrane.

The pulse recorder (Fig. 1 A) consists of a short length of wide bore brass tubing (*a*) the ends of which are closed by brass plates (*b*), grooved for the reception of the tube and clamped together by five long bolts (*c*). The back plate incorporates a shallow cup (*d*) of large diameter carrying the thick rubber membrane (*m*) with a large surface area. The front plate has a short projecting metal tube (*e*) closed by a thin rubber membrane (*t*). This membrane carries a light plane mirror mounted on a piece of cork hinging on the flat edge of the tube. A narrow bore brass side tube (*f*) and a length of narrow glass tubing serve as a compensator tube.

The ear capsule and the pulse recorder are connected by short lengths of wide glass tubing and rubber connexions (Fig. 1 B). Attached to the system is a glass syringe or pressure bottle for controlling the pressure levels: a small brass funnel carrying a thick rubber membrane and a mirror acts as a pressure recorder. The whole apparatus is filled with boiled water. The pulsations and occluding pressure levels are recorded optically on bromide paper. A signal marker cuts off a beam of light every second.

USE OF APPARATUS

Ejection of water from the syringe raises the pressure in the system and causes compression of the artery. The thick membrane of the pulse recorder bulges, driving the fluid into the compensator tube. If the pressure change is not too rapid the base line remains steady and each pulse wave is picked up by the thin outer membrane. Pulsations of the ear artery can therefore be recorded simultaneously with the occluding pressure. With rapid changes of pressure compensation is incomplete, with the result that the thin membrane also bulges


f
b
a
m
d
e
t
c
0
10
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30 mm.
f
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Fig. 1A.

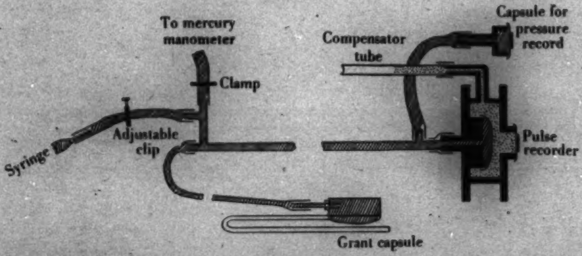
To mercury manometer
Clamp
Adjustable clip
Syringe
Compensator tube
Capsule for pressure record
Pulse recorder
Grant capsule

Fig. 1B.

Fig. 1A. Perspective and sectional diagrams of the pulse recorder. (*a*) Brass tube; (*b*) brass plate; (*c*) bolts; (*d*) shallow cup; (*e*) projecting tube; (*f*) side tube; (*m*) thick rubber membrane; (*t*) thin rubber membrane. B. General arrangement of apparatus.

forward, causing a shift of the base line. The pressure recorder is calibrated so that the record of the occluding pressure can be read in terms of mm. Hg. The optimum rate of fall of pressure must be determined for each membrane, the change being of such an order as to allow for the onset of pulsation to be clearly distinguished without causing shift of the base line. An easy control of the pressure fall was provided by a screw clamp regulating the reflux of water into the syringe.

The pulse record (Fig. 2) shows small oscillations in time with the heart beat, even when the artery under the ear capsule is obliterated by a high occluding pressure. This is caused by small vibrations of the rabbit's body, set up by each heart beat, being transmitted along the fluid of the recording system and being picked up by the recording mirror. Systolic pressure is found by drawing a vertical line through the place where there is the first obvious progressive increase of size of the recorded pulse as the occluding pressure falls. When there is difficulty in finding the place it is helpful to draw lines on the record skimming the crests of the base-line oscillations (Fig. 2). The vertical line cuts the occluding pressure line at the pressure corresponding to the systolic pressure. We could not obtain any reliable index of the diastolic pressure from these records.

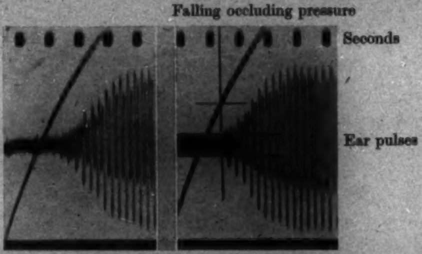

Fig. 2. Samples of recorded ear artery pulses picked up by pulse recorder. Rise of the occluding pressure line represents fall of occlusion pressure. The second sample strip shows how the 'systolic pressure point' is related to the occluding pressure line.

VALIDITY OF THE METHOD

The pressures recorded optically were compared with those obtained by the original method of Grant & Rothschild. When the optical records were being taken, a light was flashed on to the camera slit when the blood was seen to spurt along the artery. The first recorded pulse generally appeared a short time before the signal (Fig. 3). This interval was due, at least in part, to the delay between the visual observation and the signal. The measurements, however, agreed to within a few mm. Hg, and the correspondence was equally good at all pressures.

Comparisons were also made between pressures obtained from the ear artery and the carotid blood pressure (Fig. 4). At pressures of 80–100 mm. Hg the ear pressure was found to be 2–5 mm. Hg lower than the mean carotid level. This is in agreement with values found by Grant & Rothschild. At low pressures (30–50 mm. Hg), produced by bleeding, the correspondence was approximately the same in 50% of the animals. In the remainder, however, the divergence increased to 15–20 mm. Hg. Care was taken to keep the ears warm during observations, as it was realized (Grant & Rothschild) that constriction of the ear artery may cause the systolic pressure to fall 20–30 mm. Hg below the mean carotid level.

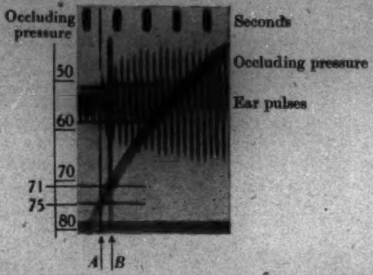

Fig. 3. Record showing relation between photographic and visual determination of systolic pressure. Ink line *A* drawn on record indicates the systolic pressure point determined photographically. Line *B* on record was made by flashing a light on to the camera slit when blood first spurted along the artery under the capsule. Occlusion pressure in mm. Hg. Photographic systolic B.P. = 75 mm. Hg; visual systolic B.P. = 71 mm. Hg.

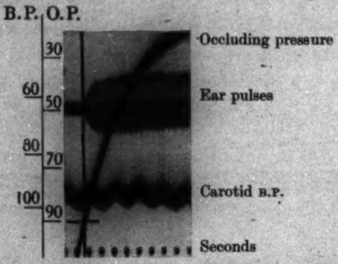

Fig. 4. Record showing relation between recorded systolic pressure in ear artery and the mean carotid blood pressure. Ear pulses recorded from right ear; carotid B.P. by cannula in left carotid artery. Urethane anaesthesia. Carotid B.P. and occluding pressure (O.P.) scales in mm. Hg. Mean carotid B.P. = 97 mm. Hg; ear artery systolic pressure = 92 mm. Hg.

Observations were also made to ascertain whether the pulsations recorded were an accurate indication of the heart rate (Fig. 5). Heart rates counted from an optical record of the carotid artery, or from an electrocardiogram, agreed exactly with the pulse waves recorded simultaneously from the ear over a wide range. Irregular rhythms, such as extrasystoles, were recorded faithfully.

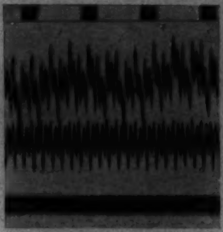

Fig. 5. Record showing correspondence between ear artery pulses, recorded from capsule on right ear, and carotid pulses, recorded from cannula in left carotid artery. Urethane anaesthesia.

TREATMENT OF THE ANIMALS

Careful control of the state of the animal is needed to ensure uniform results. It is essential that the animals remain quiet when records are being taken. Unanaesthetized animals were placed in a wooden box with the head protruding through a hole in one side. Records have also been obtained from rabbits anaesthetized with urethane (1·5–2·0 g./kg.) or nembutal (40 mg./kg. or more if needed).

The ear vessels should be fully dilated and external conditions kept as constant as possible. The animals were placed on a heated table in a warm room (17–24° C.) free from draughts. At first the ears were cold, and the vessels scarcely visible, but as the animals became warmer the ear vessels dilated and contracted rhythmically. These cycles became more prolonged and the vessels remained dilated unless the ears were handled, when the vessels constricted. Later, this irritability was lost, the vessels remaining fully dilated even when the ears were handled; venesection caused only a slight transient constriction. If the animals were not disturbed, and the ears were kept warm, the vessels remained full and prominent, even with a systolic pressure of 30 mm. Hg, following bleeding.

PREPARATION OF MEMBRANES FOR THE GRANT CAPSULE

In the original method of Grant & Rothschild, the ear capsules were closed with Cargile membrane. This was difficult to obtain at the time and a search was made for substitutes. Dried natural sausage skin proved the most useful. Wet sausage skin, as obtained from the butcher, was slipped over a boiling tube and dipped into methylated spirits for a few seconds, and then into absolute alcohol. The skin dried rapidly in air and could be opened out into a sheet. It withstood air pressure well, and was transparent when moistened with glycerol. Constant rubbing on the ear, however, caused leaks, and the skin was damaged by water. These defects were overcome by coating the skin on both sides with liquid rubber latex and allowing it to dry, or, better, by sealing a thin sheet of latex rubber to the skin with rubber solution. The skin was stretched over the neck of a flask, coated with a thin layer of rubber solution, and the latex sheet laid on top of it, any air bubbles between skin and latex sheet being worked out to the side with the fingers. The rubber sheet was not stretched but simply rested on top of the sausage skin. The preparation was left to dry for 24 hr. in a warm place. These membranes were transparent, air-tight, water-tight, and inelastic. In use the rubber side of the membrane was exposed to the water inside the ear capsule.

SUMMARY

1. A method is described for recording optically the systolic pressure and pulsations in the ear artery of the rabbit.

2. The method can be applied to both unanaesthetized and anaesthetized animals. No preliminary operation is required.

3. The relation between the peripheral and central blood pressure is discussed.

4. Directions are given for preparing inelastic transparent, waterproof membranes for use with the Grant ear capsule.

One of us (Colina Mackenzie) is indebted to the Medical Research Council for a personal grant. Part of the expenses of the investigation has been defrayed by a grant from the Government Grant Committee of the Royal Society.

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THE EFFECTS OF ACUTE HAEMORRHAGE ON THE PERIPHERAL BLOOD PRESSURE IN UNANAESTHETIZED AND IN ANAESTHETIZED RABBITS

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The consequences of haemorrhage in animals have been frequently described, but few observations have been made on the effects of a single acute loss. It is also of interest to note that investigators have paid little attention to the complicating effects of anaesthesia.

The experiments described below were undertaken to study the effects of a single acute haemorrhage on the arterial pressure and heart rate of unanaesthetized and anaesthetized rabbits.

METHOD

Adult rabbits fed on a mixed diet were used. Body weight varied between 1·4 and 3·7 kg. In early experiments the systolic pressure in the central artery of one ear was estimated by the method of Grant & Rothschild [1934]. Heart rate was counted by two observers simultaneously, using two sets of ear-pieces attached to the stethoscope bell. Later, an optical method of recording systolic pressure and pulsations of the ear artery was developed [Downman, Mackenzie & McSwiney, 1944]. The experiments were done in a warm room, and care was taken to ensure that the ear vessels were fully dilated before making observations. As the ears became warmer, the arteries dilated, but would contract if the ear was handled; later this irritability was lost, and the vessels did not respond to handling the ear [Downman *et al.* 1944]. Uniform series of pressure readings could now be obtained and there was no contraction of the arteries when the other ear was cut. Repeated observations of systolic pressure and heart rate were made to ascertain that a steady state was reached before bleeding. Unanaesthetized animals were restrained in a wooden box, with the head protruding through a hole in one side, and were not disturbed during the experiment.

In the normal blood-pressure range the systolic pressure in the dilated ear artery lies only 2–5 mm. Hg below the mean carotid pressure. At low pressures, such as occur after bleeding, differences of 20–30 mm. Hg have been observed. The pressures recorded in these experiments must, for the present, be recognized only as samples of peripheral arterial pressures.

Blood was taken from the large vein at the base of the other ear. The dorsal surface of the ear was shaved and the skin around the site of the proposed incision smeared with vaseline to prevent blood spreading. A quick cut with a sharp scalpel along the long axis of the vein divided the skin and the wall of the vessel. The blood was collected in small beakers.

To produce general anaesthesia urethane dissolved in normal saline was injected into the marginal vein of the ear. One group of animals received 1·4–1·6 g. urethane per kg. body weight; another group received 1·64–1·9 g. per kg. The effects of the different doses are described later.

RESULTS

Unanaesthetized rabbits

On cutting the vein, blood flowed rapidly for from 3 to 5 min. The flow then slowed quite suddenly; oozing continued for several minutes and then ceased entirely.

The volume of blood lost varied from 12 to 51 ml., but with one exception this did not exceed one-third of the calculated blood volume. Assuming that adult rabbits have a blood volume of 70 ml./kg. body weight [Courtice, 1943], these losses represented 7–33% of the blood volume or 0·5–2·3% of the body weight. The one exceptional animal lost 36% of its blood volume.

The animals were not obviously distressed by the haemorrhage even though nearly one-third of the blood volume was lost in 4–5 min. There was, however, an increase in the rate and depth of breathing. All unanaesthetized animals bled in this manner survived.

The ears remained warm and the main vessels showed no change of diameter to the naked eye during and after bleeding. Small areas of the ear were sketched at intervals before and after venesection; there was no apparent alteration in the size or number of the visible vessels, nor did the arterial pulse volume decrease perceptibly. There was in some animals, nevertheless, evidence of contraction of the minute vessels, shown by slight pallor of the ear tissues. This continued for some hours after bleeding.

Cessation of bleeding occurred after the initial fall of blood pressure. The pressure was recovering quickly at the time and may even have reached the initial level. Furthermore, the pressures recorded from the cut ear were the same as those in the other ear. The end-point was not influenced by intravenous heparin, nor by anaesthetizing the tissues round the incision with 2% procaine solution. These results suggest that the cessation of bleeding is not dependent primarily upon blood-pressure fall, altered blood coagulability, nor extrinsic nervous mechanisms. During and after the bleeding the vein remained dilated, except at the cut and for 2–3 mm. beyond each end of the cut. If saline was injected into the vein towards the cut, through a needle inserted distal to the cut, a pressure of 10–20 mm. Hg was needed to cause saline to flow through the constricted portion of the vein. When the vein was then occluded proximal to the cut a perfusion pressure of 100 mm. Hg was needed to open the cut. When bleeding ceased, manipulating and rubbing the cut caused no further loss of blood, but an hour later the same treatment resulted in copious bleeding. It seems that in these animals the haemostasis was produced by a powerful localized spasm of the vein wall set up and maintained by local means. The spasm passes off within the hour but meanwhile the cut edges of the vein have become stuck together. The walls can be unstuck again by manipulation.

Blood-pressure change. One to three minutes after the onset of bleeding systolic pressure in the ear artery fell steeply from the initial level of about 100 mm. Hg down to 30–40 mm. Hg. Almost at once the pressure started to rise quickly even though bleeding continued. The subsequent changes of blood pressure were of three types. In the first type, the recovery of pressure continued and pre-haemorrhage readings were obtained in 10–20 min. In the second type, the recovery of pressure approximated to only two-thirds of the initial fall in 40 min.; subsequently pressure fell again to 40 mm. Hg, and there was a slow return to the initial level. In the third type, the recovery amounted approximately to only one-third of the initial fall; later the pressure fell again and return to the initial level occupied many hours. The majority of the unanaesthetized rabbits usually showed the first type of recovery curve. A minority showed the second type, and only exceptional animals showed the third. Spontaneous falls and rises of pressure of the order of 30 mm. Hg were common after bleeding but were rare before bleeding when the animals were kept under the standard conditions described.

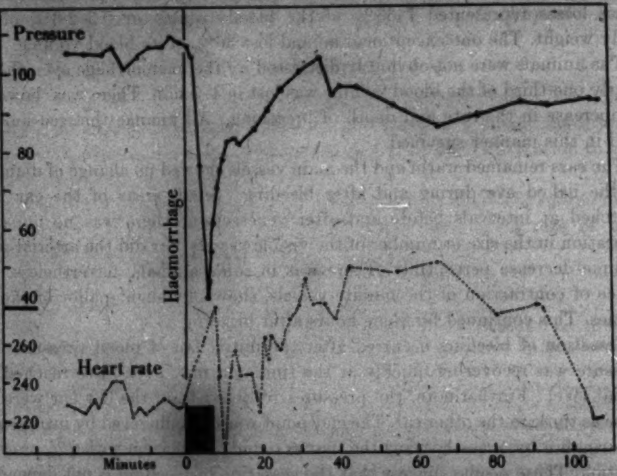

Fig. 1. Changes of systolic pressure in ear artery and of heart rate, in unanaesthetized rabbit. The animal was bled, 35 ml. in 7 min. from ear vein, equivalent to loss of 20% of blood volume. Systolic pressure in ear artery in mm. Hg. Heart rate in beats per minute.

Heart rate. With the onset of bleeding the heart accelerated rapidly. The increase of rate varied between 20 and 160 beats per min. representing rises of 8–80% over the initial rate of about 200 beats per min. After the haemorrhage had ceased, the heart rate increased further and reached a maximum about 90 min. after bleeding. The rate then declined slowly, reaching the initial level about 3 hr. after bleeding. Frequently there was a temporary decline of heart rate at the end of bleeding, the fall coinciding with the return of blood pressure. The heart rate soon increased again and the usual prolonged tachycardia was noted. An initial slowing of the heart with the onset of bleeding was never observed.

Anaesthetized rabbits

The first series of animals received 1·4–1·6 g. urethane per kg. intravenously. Corneal and other superficial reflexes were brisk, and deep reflexes very easily elicited. These animals are described as being under 'light' anaesthesia. With a loss of 23–33 % of the blood volume the early changes of systolic pressure and heart rate were similar to those previously described. The animals remained in good condition and reflexes remained brisk until the animals were killed 8 hr. later (Fig. 2).

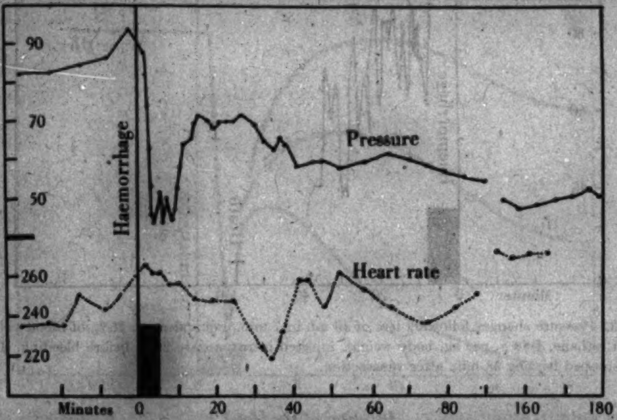

Fig. 2. Pressure changes following loss of 34 ml. of blood in 5 min., equivalent to 26 % of blood volume. Urethane, 1·50 g. per kg. body weight injected intravenously 2 hr. before bleeding. Animal killed 5 hr. after bleeding, in good general condition, with ear artery systolic pressure of 45 mm. Hg.

The second series of animals received 1·64–1·9 g. urethane per kg. intravenously. Superficial reflexes were absent and deep reflexes sluggish. These animals are described as being under 'deep' anaesthesia. With loss of 21–33 % of the blood volume, pressure fell sharply to 30–40 mm. Hg. An immediate rise of 20 mm. Hg was followed by a second decline of pressure and death in 1 to 3 hr. (Fig. 3).

Animals of a control series were anaesthetized with similar amounts of urethane. They remained in good condition without marked change of arterial pressure or pulse rate until they were killed 8–10 hr. after induction of anaesthesia.

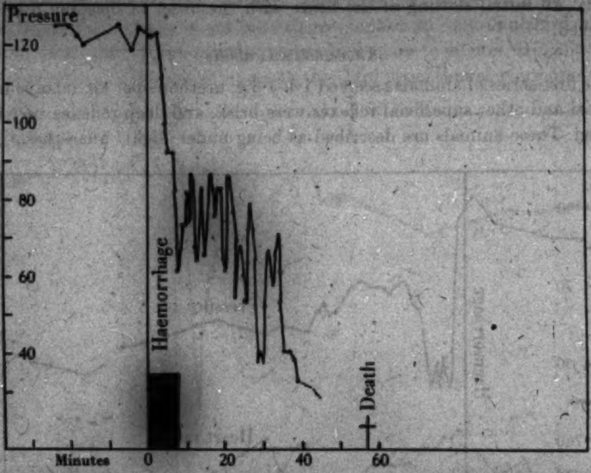

Fig. 3. Pressure changes following loss of 40 ml. in 7 min., equivalent to 28 % of blood volume. Urethane, 1·88 g. per kg. body weight, injected intravenously 2 hr. before bleeding. Heart stopped beating 58 min. after venesection.

Dilution of the blood

A few observations were made on the changes of red cell concentration in unanaesthetized and anaesthetized rabbits. Haemoglobin was estimated by the Gowers-Haldane method. Haematocrit values were determined by spinning the blood at 2500 r.p.m. for 45 min. During the bleeding, haemoglobin percentage, red cell count and packed red cell volume fell rapidly. Samples of the issuing blood showed the fall of red cell concentration, and at the end of bleeding the values represented two-thirds of the ultimate change. Following bleeding there was a further decrease of red cell concentration, complete in about 3 hr. These changes were not confined to the issuing blood but were seen in blood samples taken directly from the heart.

These observations confirm the findings of other workers. They show that the blood is 'diluted' rapidly during bleeding and more slowly for about 2–3 hr. after bleeding. It was also confirmed that the coagulation time decreased during bleeding. The last drops of blood clotted quickly in the presence of the usual amount of oxalate, and excess of oxalate did not always prevent clotting.

Discussion

The above method of bleeding was used because it was considered that the results would more nearly resemble those seen after free haemorrhage following trauma to a large vessel. The blood loss was limited by the animal's own homoiostatic mechanisms.

Unanaesthetized and 'lightly' anaesthetized rabbits suffer haemorrhage up to one-third of the blood volume without obvious distress. There was no alteration in diameter of the large vessels of the ears. No unanaesthetized animals died, and 'lightly' anaesthetized animals were in good condition 8 hr. after bleeding. On the other hand, with 'deep' anaesthesia, death occurred in 1–3 hr. after the bleeding. These results show that the injection of different quantities of a non-volatile anaesthetic, such as urethane, may considerably influence the response to bleeding.

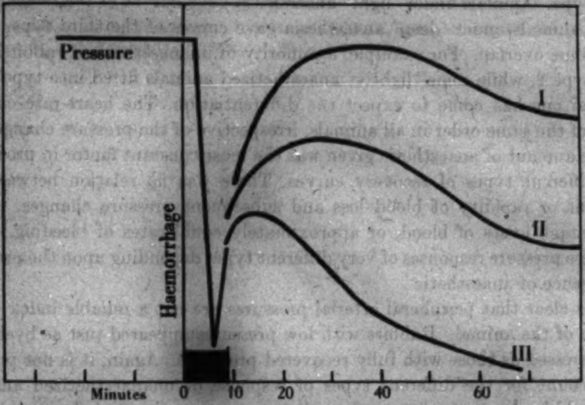

Fig. 4. Diagrammatic summary of the three types of systolic pressure change occurring in the ear artery after haemorrhage.

The adjustment of the circulation to haemorrhage, expressed in terms of blood pressure, varied in different rabbits. In all instances there was at first little or no change of pressure following venesection. After a latency of 1–3 min. the systolic pressure in the ear artery fell sharply to 30–40 mm. Hg. Thereafter pressure rose quickly, even while bleeding was in progress. The subsequent recovery curves could be differentiated into three types (Fig. 4).

Type 1. Recovery to the initial level. This was complete within 30 min. The high pressure might be maintained or show a small fall after an hour or two.

Type 2. Recovery equivalent to about two-thirds of the initial fall, but a decline again after 40 min. to a lower level. The pressure then rose slowly towards the initial level.

Type 3. A short-lived rise equivalent to about one-third of the initial fall. This recovery was not maintained, pressure falling quickly again to 30–40 mm. Hg. Any later recovery of pressure was very slow.

The consequence of bleeding could be related to the depth of urethane anaesthesia. Unanaesthetized animals usually gave recovery curves of the first type. Animals under 'light' anaesthesia gave curves of the second type, while animals under 'deep' anaesthesia gave curves of the third type. There was some overlap. For example, a minority of unanaesthetized rabbits fitted into type 2, while some 'lightly' anaesthetized animals fitted into type 1. In general one has come to expect the differentiation. The heart-rate changes were of the same order in all animals, irrespective of the pressure changes.

The amount of anaesthetic given was the most constant factor in producing the different types of recovery curves. There was no relation between the amount or rapidity of blood loss and subsequent pressure changes. Equal percentage losses of blood, or approximately equal rates of bleeding, might produce pressure responses of very different types depending upon the presence or absence of anaesthetic.

It is clear that peripheral arterial pressures are not a reliable index of the future of the animal. Rabbits with low pressures appeared just as lively and undistressed as those with fully recovered pressures. Again, it is not possible to account for the different types of response of unanaesthetized animals. It should be emphasized that, since all experiments were carried out under the same conditions, it seems that the differences in response depend upon the physiological characteristics of the animal concerned. Anaesthetics, on the other hand, may upset the normal sequences of the adaptation forces and so alter the blood-pressure recovery.

It would be premature to compare these results with clinical findings because the experiments have been confined to rabbits and only urethane has been used as anaesthetic. Furthermore, the rapid dilution of the blood in the rabbit contrasts with the slow dilution in man.

SUMMARY

1. Unanaesthetized rabbits, and rabbits 'lightly' anaesthetized with urethane, may lose up to one-third of the blood volume by rapid free bleeding from a vein of the ear without distress or deterioration of condition.

2. Animals under 'deep' urethane anaesthesia die 1 to 3 hr. after bleeding.

3. The recovery curves of systolic pressure in the ear artery could be differentiated into three types. The dose of anaesthetic could be related to the type of recovery curve found.

4. Increase of heart rate persisted up to 3 hr. after bleeding and long outlasted the pressure changes.

One of us (Colina Mackenzie) is indebted to the Medical Research Council for a personal grant. Part of the expenses of the investigation have been defrayed by a grant from the Government Grant Committee of the Royal Society.

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Grant, R. T. & Rothschild, P. [1934]. *J. Physiol.* 81, 265.

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GLYCOGEN IN ADIPOSE TISSUE

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When rats are fed on a carbohydrate-rich diet after prolonged starvation, appearance of glycogen within adipose tissue is an early indication that deposition of fat is impending. Other findings also suggest that glycogen is converted in the adipose tissue into fat (Tuerkischer & Wertheimer, 1942; Mirski, 1942). On this view it is expected that glycogen will precede fat deposition in adipose tissue whenever newly synthesized fat, derived from carbohydrate, is stored. The present paper describes the continuation of experiments on the relation between glycogen and fat within adipose tissue.

I. *Insulin treatment.* Clinicians have long assumed that insulin favours deposition of fat. The effect is generally believed to be an indirect one due to a stimulation of the appetite. Drury (1940) and MacKay, Callaway & Barnes (1940) showed that insulin increased appetite, body weight, and fat storage in rats and rabbits. Is such fat deposition under insulin treatment preceded by deposition of glycogen within the adipose tissue? Is the insulin effect direct, or only secondary to increase in appetite for carbohydrate? As early as in 1927 Hoffmann & Wertheimer demonstrated that insulin sometimes induces glycogen deposition in the adipose tissue of dogs. Insulin was found to be without effect on glycogen deposition in the adipose tissue of rats fed a carbohydrate-rich diet after being starved (Tuerkischer & Wertheimer, 1942).

II. *Alternate starving and feeding.* MacKay & Drury (1941) have shown that, when rats are fed on a diet consisting almost entirely of carbohydrates and starved on alternate days, so that their average body weight and food intake become constant, the food carbohydrate comes to be stored largely in the form of fat. Is fat deposition in this case also associated with the appearance of glycogen in adipose tissue?

III. *Carbohydrate excess.* Is glycogen deposited in the adipose tissue after excessive intake of carbohydrates?

METHODS

The rats used were of laboratory stock. As a rule, young male animals weighing 80-110 g. were selected. They were maintained on a standard diet consisting of wheat and vegetables or on a carbohydrate-rich synthetic diet (70% carbohydrate, 20% casein, 10% fat with the usual supplements of minerals and vitamins). In order to make sure that the rats were showing a regular increase in weight, they were weighed daily for about a week before their use in an experiment. The room temperature was not allowed to fall below 20–21° C. and large fluctuations were carefully avoided. Glycogen deposition in adipose tissue following insulin administration was easier to demonstrate in summer than in winter. In winter experiments a higher insulin dose was found to be necessary. Protamine-Zinc-Insulin (PZIns.) was more effective than ordinary insulin.

The following conditions proved suitable: the rats were given a first dose of PZIns. in the morning. After 3–4 hr. a second injection was given. Six to eight hours after the first insulin injection the rats were killed.

The adipose tissue was treated in the manner described by Tuerkischer & Wertheimer (1942). Groin, testicle and perinephric fat were pooled and weighed and are referred to as 'mixed fat'. Fat from mesentery and brown interscapular fat were weighed and treated separately (Hook & Barron, 1941). For glycogen determination, samples were taken as follows: 'mixed' fat 0·5 g., mesentery fat 0·2 g., interscapular fat 0·1–0·2 g. Chemical procedures employed have been already described (Tuerkischer & Wertheimer, 1942). Glycogen values are given as g. glucose per 100 g. fresh tissue.

The experiments in series 1, 2 and 3 were carried out in summer, those in series 4 to 7 in winter and spring at a room temperature of 22 to 23° C.

1. In this series of 11 experiments the rats were given 25 % glucose solution to drink during the 24 hr. period before insulin injection. During the time of insulin action normal diet and tap water were allowed. The dose of insulin was 2 × 0·3 units PZIns. Included in this series were 4 experiments comparing the reaction of young and old rats. The old rats had an average weight of 180 g.
2. In 10 experiments the rats received 3·5 c.c. 25 % glucose by stomach tube just before the first insulin injection. Otherwise the experimental conditions were as in series 1.
3. In 19 experiments the rats were given 25 % glucose solution to drink during the experimental period. The other experimental conditions were unchanged.
4. In 11 experiments the conditions were as in series 3 but the dose of insulin was 2 × 3 units PZIns.
5. In 19 experiments the rats received only tap water to drink and the dose of PZIns. was 2 × 1 units.
6. In 13 experiments the conditions were as in series 5 but the rats were on three different diets: (*a*) 70 % casein, 20 % carbohydrate, 10 % fat with the usual supplements; (*b*) 50 % fat, 25 % carbohydrate, 25 % casein; (*c*) 70 % casein, 30 % fat.
7. In 5 experiments the conditions were as in series 6 (*a*) but the insulin dose of 2 × 0·3 units PZIns. was given after a 20 hr. fast. The rats were killed 5 hr. later.

RESULTS

I. *Influence of insulin on glycogen deposition in adipose tissue*

Table 1 gives a typical result for series 1 to 6 (*a*) inclusive. In 6 (*b*), 6 (*c*) and in 7 glycogen was found only in the interscapular fat. When insulin administration (2–6 units PZIns. daily in winter experiments) was prolonged for from 2 to 9 days, and normal diet was allowed, the occurrence of glycogen in adipose tissue became a constant feature. This was observed in 36 experiments. In similar experiments with rats maintained on the 70 % casein diet, glycogen deposition in the adipose tissue of the same order was observed only on the first and second days of the experiment.

The rise and fall of the glycogen level in adipose tissue was investigated in 275 experiments. Glycogen deposition appears in the interscapular fat as

TABLE 1. Glycogen deposition in adipose tissue after administration of protamine-zinc-insulin

	Intake of glucose sol. (c.c.)	Glycogen, g./100 g., in			Blood sugar mg./100 c.c.
		Interscap. fat	Mesent. fat	Mixed fat	
Insulin	23	0·30	0·21	0·11	71
Control	27	0·03	Traces	Traces	112

early as about ¾ hr. after administration of 1–3 units PZIns. The curve of the glycogen deposition rises with the fall of the blood sugar, reaches its peak at about 10–14 hr., and falls to zero again after 16–20 hr. with the return of the blood sugar to normal. Glycogen deposition in the 'mixed' and in the mesentery fat begins only at 4 hr. after insulin administration, and follows in its rise and fall the curve of the glycogen deposition in the interscapular fat. The glycogen values in the 'mixed' and the mesentery fat were much smaller than in the interscapular fat. With normal insulin the glycogen curves returned to zero sooner than with PZIns.

All time-curves on glycogen deposition were carried out in summer experiments.

Control tests were carried out at different times on 45 untreated rats. The values obtained in the controls for 'mixed' fat and for mesentery fat were uniformly nil; for interscapular fat a single value of 0·05% and one of 0·2% were recorded. The findings, with data published in our earlier paper (Tuerkischer & Wertheimer, 1942), support the conclusion that, in normal conditions, glycogen very rarely occurs in adipose tissue.

Do carbohydrates other than glycogen appear in adipose tissue following insulin injection? Both glycogen and total carbohydrate were determined in adipose tissues following insulin injection. The method of West, Scharles & Peterson (1929) was employed. Analyses were carried out on 'mixed' fat both before and after glycogen begins to appear as the result of insulin injection. In all cases the carbohydrate was present as glycogen only.

Glycogen in adipose tissues of herbivores after insulin treatment. In rabbits and guinea-pigs maintained on an ordinary diet (bran, oats, vegetables), insulin treatments (3–12 units PZIns. per kg.) failed to induce glycogen deposition in the adipose tissues. MacKay *et al.* (1940) have emphasized that 'the rabbit is not a very good animal for the type of experiment to gain much weight with insulin administration. The reason for this is the low energy value of its food when calories per unit volume are considered'. According to Long & Bischoff (1930) insulin treatment does not induce increase of body weight in rabbits.

If, however, rabbits are given a diet rich in concentrated carbohydrates, a very small deposition of glycogen can be observed in the adipose tissue. Following insulin administration the amount of glycogen in the adipose tissue is doubled.

The following procedure was found most suitable: small thin rabbits weighing about 1 kg. were given 30–40 c.c. 25 % glucose solution by stomach tube and 10 units PZIns. per kg. body weight in the morning; 3 hr. later the insulin injection was repeated. After 5–7 hr. the animals were killed.

In fat animals the glycogen values were lower. In two cases no definite difference between the experimental animal and the control could be established. The results of the analyses are summarized in Table 2.

Table 2. Mean values for adipose tissue glycogen in rabbits treated with protamine-zinc-insulin

	No. of expts.	Glycogen, g./100 g., in				Blood sugar mg./100 c.c.
		Interscap. fat	Mesent. fat	Mixed fat	Liver	
Insulin	14	0·12±0·010	0·09±0·017	0·10±0·007	7·0	97
Controls	15	0·06±0·007	0·05±0·009	0·05±0·006	7·3	128

II. *The effect of alternate starving and feeding*

Rats were subjected to the MacKay & Drury regimen of alternate starving and feeding. Animals which failed to maintain their weight were eliminated from the tested group. Experiments were performed in winter. Table 3 gives the results.

Table 3. Glycogen in adipose tissues of rats maintained on a McKay-Drury regimen and killed 6 hr. after recovery feeding

Mean values for 3 animals in each case

No. of fast days	Glycogen, g./100 g., in		
	Interscap. fat	Mesentery fat	Mixed fat
1	0·52±0·16	0·10±0·02	0·08±0·03
2	2·20±0·59	0·21±0·09	0·25±0·07
3	2·64±0·35	0·35±0·07	0·23±0·10
4	2·48±0·56	0·52±0·09	0·33±0·11
5	2·70±0·35	0·55±0·18	0·56±0·12
6/7	1·12±0·24	0·52±0·17	0·17±0·05

In order to time the glycogen deposition, groups of rats which had completed their fourth fast day on the MacKay & Drury regimen were killed at different times after feeding. The results of an experiment carried out in winter are presented in Table 4.

Table 4. Glycogen in adipose tissues of rats killed at different times after recovery feeding in the MacKay-Drury test

Mean values for 3 animals in each case

Time after recovery feeding	Glycogen, g./100 g., in		
	Interscap. fat	Mesentery fat	Mixed fat
30 min.	0·14±0·04	0	0
1 hr.	0·70±0·16	0	0
2 hr.	0·96±0·08	0	0
4 hr.	1·90±0·12	0·23±0·08	0·08±0·01
6 hr.	2·15±0·38	0·52±0·15	0·22±0·10
1 day	0·61±0·11	0·26±0·08	0·37±0·17
2 days	0·40±0·05	0·21±0·06	0·23±0·05
3 days	0·50±0·28	0·08±0·03	0·08±0·02
4 days	0	0	0

In summer, higher values were obtained. In five summer experiments, after 4 days fast and in the sixth hour after feeding, glycogen values (g./100 g.) were found as follows: 'mixed' fat 0·58, mesentery fat 0·82, interscapular fat 4·3.

If animals in the MacKay-Drury test after 4 days fast are fed and then again starved, a rapid disappearance of glycogen from the adipose tissues occurs. Following 6 hr. of fast after 6 hr. of feeding, glycogen values (g./100 g.) were obtained as follows: 'mixed' fat 0·15, mesentery fat 0·06, interscapular fat 0. If the fast was prolonged to 12 hr. all the values were zero. There were three rats in each experiment. Experiments showing the effect of different duration of fast and of feeding on glycogen in adipose tissue are assembled in Table 5.

Table 5. Glycogen deposition in adipose tissue after different fast and feeding periods
Mean values for 3 animals in each case

Fast period hr.	Hours after end of starvation	Glycogen, g./100 g., in		
		Interscap. fat	Mesentery fat	Mixed fat
24	6	0·53 ± 0·10	0·10 ± 0·04	0·08 ± 0·03
	24	0·39 ± 0·08	0·08 ± 0·04	0·16 ± 0·03
	32	0·09 ± 0·05	0·08 ± 0·03	0
10	6	0·40 ± 0·04	0·22 ± 0·05	0·08 ± 0·03
	24	0	0	0
6	6 and 24	0	0	0

In all the experiments the fast periods were arranged so that they occurred at night. The findings in experiments according to MacKay & Drury (1941), as well as those with prolonged hunger (Tuerkischer & Wertheimer, 1942), support the conclusion that the quantity and duration of the glycogen deposition in the adipose tissues following feeding vary positively with the length of the preceding hunger period, i.e. with the amount of the fat loss and the corresponding increase in fat-storage capacity.

In the MacKay-Drury test, as well as in the earlier experiments of Tuerkischer & Wertheimer (1942), fat of adrenalectomized rats in good condition was found to be practically free from glycogen. Adipose tissue glycogen deposition in rats kept on a B_1-free diet is diminished.

III. *Carbohydrate excess*

A brief period of glycogen deposition in the adipose tissue can be distinguished in rats maintained on a diet excessive in carbohydrates and which are not starved at all. Carbohydrate saturation is most easily effected by giving rats, on an ordinary diet, 25% glucose solution, rather than water, to drink. The clearest results were obtained in experiments of 6–8 hr. duration. The majority of the experiments were performed in winter. The results are shown in Table 6.

Table 6. Mean values for glycogen in adipose tissue after excess intake of carbohydrate

No. of expts.	Intake of sugar sol. c.c.	Glycogen, g./100 g., in Interscap. fat	Mesentery fat	Mixed fat	Blood sugar mg./100 c.c.
15	15	0·24±0·05	0·15±0·03	0·17±0·04	119

After excess intake of carbohydrate had been continued for 12–24 hr. glycogen was rarely detected in adipose tissue. In summer the amount of glycogen deposition in the adipose tissues following excess intake of carbohydrates was smaller.

DISCUSSION

Tuerkischer & Wertheimer (1942) suggested that glycogen *always* accumulates in adipose tissue whenever synthesis of fat from carbohydrate occurs. The simplest example of this is found in chronic underfeeding followed by recovery-feeding with carbohydrate. Further examples have been described in the present communication. MacKay & Drury (1941) found that animals, which are alternately starved and fed, store carbohydrate temporarily as fat. This storage too is associated with deposition of glycogen in adipose tissues. A fleeting deposition of glycogen in adipose tissue can be induced even without preceding fasting if a large excess of carbohydrates is fed.

MacKay, Barnes & Carne (1941) showed that rats fed on a carbohydrate-rich concentrated diet, and treated with Protamine-Zinc-Insulin, react with marked increase in appetite, in body weight and in fat deposition. In this condition of fat synthesis from carbohydrate, glycogen also appears within the adipose tissues. The findings seem to be best explained by the assumption that glycogen is an intermediary in fat synthesis from carbohydrate in adipose tissues.

A further question to be considered is whether the effect of insulin on the fatty tissue is direct or indirect. The marked increase in appetite which insulin produces might suggest that insulin acts merely by increasing carbohydrate intake. This conclusion is at variance, however, with the following: 1. The effect of insulin is frequently demonstrable in the interscapular fat within about 45 min. of insulin injection, i.e. before any effect on appetite has become apparent and certainly before any such effect could become of importance. 2. MacKay *et al.* (1941) have shown that insulin fails to induce increased appetite and food intake in rats maintained on a protein-rich diet; nevertheless, glycogen is deposited in the adipose tissues in these conditions. It occurs, moreover, in the interscapular fat also in rats starved for 24 hr. after protein rich feeding. 3. In adrenalectomized animals, insulin fails to increase the carbohydrate appetite. Nevertheless, glycogen deposition in the adipose tissue of adrenalectomized rats, following insulin injection, is especially marked. It is proposed to discuss this result further in a succeeding communication.

It may be concluded therefore that the influence of insulin is primary and direct on glycogen synthesis by the adipose tissue. The reactivity in this respect is a new proof of the active role of the adipose tissue in the carbohydrate-fat exchange. On quite different grounds Longenecker (1941) writes: 'The recognition that the fat depots are centres of continuous metabolic activity represents a fundamental change in the conception of a tissue which previously had been considered inert.'

A third question to be considered is whether the present findings have any special bearing on the role of insulin in carbohydrate balance. Glycogen disappears from the adipose tissue simultaneously with the disappearance of the insulin effect on the blood sugar level and reappears only following a renewed insulin stimulus. It has also been shown that prolonged insulin action induces an increase in the fat deposits. Experiments *in vitro* by Mirski (1942) give strong support to the view that glycogen within the adipose tissue is converted into oxygen-poor substances, probably fatty acids. We assume that the amount of glycogen found in the adipose tissue is merely the resultant of simultaneously proceeding processes of both synthesis and breakdown. The conversion of carbohydrate into fat, a uniform accompaniment of a carbohydrate-rich diet, has not yet been given sufficient consideration. Pauls & Drury (1942) concluded as follows: 'Most ingested carbohydrate is normally stored as fat, and it is considered the most likely possibility that this is a fate of the bulk of the sugar stored under the influence of insulin.'

Schur, Loew & Krcma (1934), even before this, pointed out the role of insulin as a hormone regulator of nutrient storage. The few experiments carried out, however, did not prove this conclusion.

SUMMARY

1. In suitable conditions, insulin induces synthesis of glycogen in the adipose tissues of the rat. The synthesis is particularly rapid and marked in the brown interscapular fat. Glycogen disappears from fatty tissues simultaneously with the disappearance of the insulin effect on the blood sugar level. Synthesis of glycogen in adipose tissues can also be demonstrated in the rabbit, but is less marked.

2. It is shown that insulin directly affects synthesis of glycogen in adipose tissue and does not act through an effect on carbohydrate food intake.

3. It is considered that synthesis of glycogen in adipose tissues, under the influence of insulin, is a primary step in the transformation of carbohydrate into fat.

4. In animals which are alternately starved and fed (MacKay & Drury, 1941), deposition of fat has been found to be preceded by deposition of glycogen in the adipose tissues.

5. In animals first starved and then fed, the amount and the duration of glycogen deposition in the fat tissues depend on the length of the fast period.

6. In rats, which have not been starved, administration of an excess of carbohydrate frequently induces brief deposition of glycogen in fatty tissues.

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SYNTHESIS OF ACETYLCHOLINE BY TISSUE OF THE CENTRAL NERVOUS SYSTEM

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This paper deals with the problem of synthesis of acetylcholine by brain tissue. The tissue has been examined after grinding in the fresh condition and after drying; in both cases it has been suspended in saline solution with or without the addition of ether and with the addition of eserine. Under these conditions the effects on acetylcholine synthesis of temperature, glucose, iodoacetic acid, oxygen, cyanide, potassium and calcium ions have been observed.

Synthesis of acetylcholine by brain tissue has been described by Quastel, Tennenbaum & Wheatley [1936] and by Mann, Tennenbaum & Quastel [1938, 1939]. They used brain slices or a suspension of minced brain incubated in saline solution with eserine. They were led to the conclusion that a relationship existed between tissue respiration and the synthesis of acetylcholine, suggesting that the latter might be dependent on the metabolism of the living cells. On the other hand, Stedman & Stedman [1937, 1939] observed synthesis of acetylcholine in a suspension of minced brain, incubated in a saline medium containing excess of chloroform as well as eserine, and later in one containing eserine and ether; under these conditions the metabolism of the living cells could not be concerned. To throw further light on this question of the synthesis of acetylcholine by brain substance which could no longer be regarded as living, respiring tissue, brain substance which has been dried and powdered has been examined.

Many of the experiments have been directed towards the examination of the storage by fresh brain tissue, or dried powder, of synthesized acetylcholine in varying amounts and, particularly, to discover whether the store can increase beyond the acetylcholine complement of fresh brain tissue. The acetylcholine is held in the tissue bound to some constituent, and is, in this condition, relatively immune to the action of the cholinesterase. Mann *et al.* showed that the acetylcholine, as produced by synthesis, first appears bound to the tissue, and is then released into the solution from tissue slices or, when a suspension of minced brain is used, from cell debris or even whole cells. No synthesis was observed by them in the supernatant fluid after the particulate matter had been removed by the centrifuge. Recently it has been found [Feldberg, 1943] that synthesis of acetylcholine in the tissue of a peripheral nerve or a sympathetic ganglion suspended in saline, occurs only at a rate sufficient to replace what is released from the tissue into the solution. This tissue is unable to store acetylcholine in excess of its physiological complement.

METHODS

Cats, dogs, rabbits and guinea-pigs were used. The cats and dogs were anaesthetized with ether-chloroform and killed by bleeding from the carotid artery. The rabbits and guinea-pigs were killed by a blow on the neck.

Certain abbreviated descriptions are needed in this paper and are to be read with the following meaning. *Brain:* The material was, in all cases, brain tissue anterior to a section across the brain stem between the anterior and posterior corpora quadrigemina. It includes therefore the cerebral hemispheres, basal ganglia and the part of the stem anterior to the section. *Half-brain:* Half of the above, obtained by median longitudinal section giving two completely symmetrical portions. *Saline:* 9 g. NaCl, 0·42 g. KCl and 0·24 g. $CaCl_2$, made up with distilled water to 1000 c.c.; to this solution 300 c.c. *M*/15 sodium phosphate buffer were added (*p*H about 7·3). When larger amounts of KCl or $CaCl_2$ or of glucose were used, the NaCl of the saline was reduced to keep it isotonic. *Eserine-saline:* The above saline with the addition of 0·005–0·02 % eserine sulphate.

Acetylcholine content of fresh brain. Most determinations were made with guinea-pigs' brains, which weighed between 2·2 and 3·3 g. The brains were ground with silica in a mortar with 2 c.c. *N*/3 HCl per brain and a few c.c. of eserine-saline. The suspensions were transferred to flasks, further diluted with saline, boiled and then cooled. In this condition they could be kept in the cold for a day or two without perceptible change in the acetylcholine content. Usually, however, the assay was carried out on the same day. The extracts were filtered, the residue washed with a few c.c. of saline and the filtrate neutralized with *N*/3 NaOH. Distilled water was added in a proportion of 1 to 3 to render the solution isotonic with frog saline. Finally, the diluted filtrate was made up with frog saline to such volume that 1 c.c. was equivalent to 50 or 100 mg. of fresh tissue, and from this the required dilutions were made for assay on the frog's rectus preparation. Details are given in the text.

Incubation of fresh brain tissue of the guinea-pig. For incubation in eserine-saline a half-brain was ground in this solution with silica in a mortar, and transferred to a flask which was shaken in a water-bath at the required temperature. The volumes of solution used are given in the text. During the period of incubation, oxygen was bubbled through the suspension. After the incubation, *N*/3 HCl (about 1 c.c. for each half-brain) was added to the samples which were then boiled and treated as above. To determine the acetylcholine in solution, separately from that held by the tissue particles, the incubation was carried out in wide centrifuge tubes. After incubation, the particulate matter was spun down, the supernatant fluid removed, and HCl added both to it and to the residue, which was resuspended in eserine-saline. Residue and supernatant fluid were then treated and assayed separately.

After incubation in eserine-saline with ether, the samples were acidified with *N*/3 HCl, and the ether evaporated on a water-bath before treatment as above. Details are given in the text. When samples containing ether were incubated at 36° C., the ether lost by evaporation was replaced every 10 min.

Drying nervous tissue. The brain immediately after removal from the animal was weighed and cut into thin slices which were spread on a Petri dish and dried over P_2O_5 *in vacuo* at about 2° C. The material was removed every few days and cut into smaller pieces to accelerate drying until, finally, after 4–5 days, it could be powdered and then kept in the vacuum desiccator at 2° C. till required. 1 g. of fresh tissue yielded roughly 220 mg. of this powder.

RESULTS

Assay of acetylcholine. The fact that acetylcholine has been identified chemically in extracts of brain tissue [Stedman & Stedman, 1937] made unnecessary a detailed pharmacological comparison of the actions of the extracts with those of acetylcholine. In addition to their action on the rectus muscle of the frog, they produced in the cat the expected fall of arterial pressure, which was abolished by atropine. The active constituent of the extracts also resembled acetylcholine in sensitivity to alkali and to cholinesterase.

The acetylcholine content of a brain extract as determined by physiological assay against pure acetylcholine appears somewhat greater, however, than the true value. Brain extracts, in fact, contain a principle which renders the frog muscle sensitive to acetylcholine. This can be shown as follows. Brain extract was boiled in alkaline solution to destroy any acetylcholine present; the extract was cooled, neutralized and diluted; a known dose of acetylcholine was then added. The effect of such a solution on the rectus muscle was stronger than that of the same dose of acetylcholine given without extract. The extract had no action on the muscle by itself. Under such conditions, inactivated extracts of the guinea-pig's brain equivalent to 10 mg. of fresh tissue, increased the response to acetylcholine by 10–20%; with stronger extracts the difference was even greater. In all experiments, therefore, the extracts were assayed against acetylcholine solutions to which had been added equivalent amounts of such inactivated brain extracts. This procedure proved satisfactory under all conditions but one; extracts of dried brain, incubated in eserine-saline and treated in this way, developed on standing a sensitizing property which rendered the assay by means of the frog muscle untrustworthy. The responses to a given amount of acetylcholine in such an extract became stronger with repeated administration and, after a few tests, the muscle remained partially contracted, even when the fluid was replaced by fresh saline. This was avoided when the control brain extract was inactivated, not by boiling, but by keeping with alkali at room temperature for a few minutes and then neutralizing. Even so, trustworthy results were obtained only when this treatment was carried out immediately before each test.

Acetylcholine content of the fresh brain of the guinea-pig. The right and left halves of the same brain, when extracted and assayed separately, gave practically the same amounts of acetylcholine. The difference was not more than 5%. There were variations, however, between different brains. In the course of the experiments, forty-five values were obtained from separate guinea-pigs, and they varied between 2·7 and 4·5μg./g. In Fig. 1 the acetylcholine content of fresh brain tissue is plotted against the body weight of the animals. Despite the overlapping of the values, there is a general increase with body weight or age. The average value for the eighteen animals weighing less than 400 g. was 3·2μg./g., that for the twenty-seven weighing more than 400 g. was 3·7μg./g. Where no direct control was used, these basal values were assumed for subtraction, according to the weight of the animal used. In many later experiments, as in those shown in Table 1, the right half of the brain from one guinea-pig, and the left from another were used together for the control extract and the other halves for the experiment.

That no loss of acetylcholine, due to cholinesterase activity, occurs in the brain between death and extraction, was shown as follows. After a blow on the neck, the thorax was opened, a cannula was tied through the still beating heart into the aorta, and the head was perfused with Locke's solution containing 0·001% eserine. After about 15 min. the skull was opened, without inter-

TABLE 1. Synthesis of acetylcholine with ground brain of the guinea-pig, incubated for 90 min. at 36° C. in eserine-saline

c.c. saline/g. tissue	Acetylcholine in μg./g. Control	Acetylcholine in μg./g. Incubated	% increase
5·1	3·26	12·25	275
5·0	3·20	11·90	272
5·6	3·48	11·74	237
5·3	3·40	10·96	222
5·5	3·80	10·76	183
5·0	4·00	11·20	180
5·0	3·75	10·46	179
4·7	3·75	10·40	177
Average	3·58	11·21	216

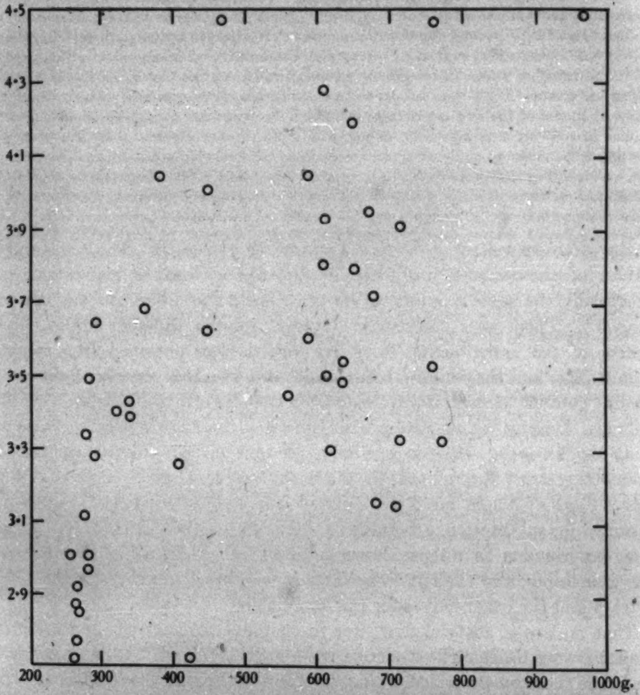

Fig. 1. Acetylcholine content of the fresh brain of the guinea-pig. Ordinates: acetylcholine in μg./g. Abscissae: body weight in g.

rupting the perfusion, and the brain was removed. The acetylcholine values of three such experiments, using for each two animals weighing over 400 g., were 3·87, 3·45, and 3·40μg./g. brain. These values are not higher than those obtained when the brains are extracted without such protective perfusion (compare the control values in Table 1).

When similar perfusion experiments were performed without eserine in the perfusion fluid, the acetylcholine content was definitely lower. In three such experiments, for which again six large animals were used, the values were 3·12, 2·41 and 2·25μg./g. (average 2·59μg./g.). Thus, the acetylcholine is not wholly protected against the tissue cholinesterase when the brain is perfused with saline solution.

I. FORMATION OF ACETYLCHOLINE BY FRESH BRAIN OF THE GUINEA-PIG

When a half-brain was incubated in about 5 c.c./g. eserine-saline for 90 min. at 36° C. it showed an increase of acetylcholine over the control half (Table 1), amounting to between 177 and 275% (average 216%). The amounts synthesized lay between 6·65 and 8·99μg./g. (average 7·63μg./g.). In these and subsequent experiments, the samples, during incubation, were continuously agitated in a mechanical shaker. In earlier experiments, the flasks had been shaken by hand only for a few moments every 5 min. or so, and the amounts of acetylcholine formed were smaller. The results of the first four experiments of Table 2 were obtained in this way.

TABLE 2. Distribution of acetylcholine in samples of incubated ground half-brains of the guinea-pig

	Acetylcholine in μg./g.			
		Incubated half		
No. of Exp.	Control half (a)	Residue (b)	Supernatant (c)	% increase of (b) over (a)
1	3·50	3·57	2·98	2
2	3·48	3·74	2·25	8
3	3·95	4·33	3·60	10
4	3·48	4·50	3·50	29
5	3·60	3·57	7·33	−1
6	3·75	4·76	4·65 + 1·05	27
7	4·00	4·25	6·25 + 0·70	6
8	3·75	3·57	6·83	−5

Distribution of acetylcholine. Samples were centrifuged after incubation, and the residue and supernatant fluid were extracted and assayed separately. It was found that the increase of about 213% in the experiments of Table 1 was chiefly due to acetylcholine in the supernatant fluid, but that the residue, as thus separated, yielded also about 50% more acetylcholine than the control half-brain, i.e. about a quarter of the total increase was found in this residue. This finding, however, is not incompatible with the supposition that the increase may be wholly due to acetylcholine passing into the fluid phase of the suspension, synthesis in the tissue particles keeping pace only with the loss by liberation and diffusion, so as to maintain the tissue content at its original level. After mere separation by the centrifuge, the deposit still contains fluid in its interstices to the extent of about 1 c.c. for each gram of original tissue; and the acetylcholine content of this interstitial fluid may well be somewhat higher than that of the free supernatant fluid, at any moment. This supposition is, indeed, supported by several observations. If passage into the fluid is not encouraged by constant shaking during the incubation, the small increase of acetylcholine in the supernatant fluid which then occurs is associated with only a small increase in the deposit (Table 2, Exps. 1–4). Even with constant shaking and a consequent large total increase of acetylcholine in the system, the increase in the deposit may be largely or wholly eliminated in one of two ways: (1) The material may be suspended for incubation in a large volume of eserine-saline (Table 2, Exp. 5), so that a large increase in the total acetylcholine of the system causes a relatively small increase of the concentration in the fluid, interstitial as well as supernatant; for this purpose glucose (1·5 mg./c.c.) must be added (see p. 376) to prevent its dilution below the level needed for synthesis. (2) The deposit from the first centrifugation may be washed by resuspension in cold eserine-saline and spun again, the second supernatant fluid being added to the first (Exp. 8) or assayed separately (Exps. 6 and 7). Under any of these conditions, it is found that the acetylcholine present in the final layer of tissue particles differs little from that found in the control tissue; the synthesis would, therefore, appear to proceed at such a rate as to maintain the store in the tissue practically constant, irrespective of the total amount which has been synthesized and liberated into the fluid under the protection of eserine. The result is similar to that already described for sympathetic ganglia and cholinergic nerves, when similarly treated [Feldberg, 1943]. In the case of the brain, however, it is not clear, as it is in the nerve or ganglion, whether the artificial conditions simply cause liberation from the tissue store, synthesis being evoked by the threat of depletion, or whether, on the other hand, synthesis is, in this case, always in action and liberation is due to overflow from the depot as soon as this is filled to the normal level. Analogy would obviously favour the former supposition.

When one half-brain is incubated for 90 min. in saline, without eserine, it yields on extraction a little less acetylcholine than the control half. In one experiment, the incubated half yielded 3·64μg./g., the control 4·01μg./g. Since all acetylcholine once released is destroyed by the cholinesterase, the 3·64μg./g. represents the acetylcholine of the particulate matter which is protected against the cholinesterase. The experiment again suggests a tendency of the brain tissue to retain or to replace its normal complement of acetylcholine.

Effect of grinding brain or finely dividing it with scissors

When brain is ground or minced in saline, without eserine, the sample yields, even without incubation, only a little less acetylcholine than a control half-brain, ground immediately in HCl (Table 3, Exp. 1). Since any acetylcholine released is at once destroyed, the 3·54μg./g. obtained in this experiment must be attributed entirely to acetylcholine held in the tissue particles, or immediately synthesized to compensate for release.

Table 3. Effect on acetylcholine yield of grinding or mincing brain tissue of guinea-pig

	Acetylcholine yield in μg./g.					
		(b) Ground in saline				
No. of exp.	(a) Control	Particulate matter	Fluid	Total	% difference	Treatment of (b)
1	4·17	—	—	3·54	−20	Ground 1½ min., no eserine
2	3·79	—	—	4·05	+ 7	Ground 3 min.
3	3·60	—	—	4·49	+28	Ground 3 min.
4	3·54	—	—	4·42	+25	Finely divided for 8 min.
5	3·72	—	—	4·83	+31	Finely divided for 8 min.
6	3·00	2·91	0·90	3·81	+27	Ground 1½ min. Centrifuged
7	3·16	3·33	0·72	4·05	+28	Ground 1½ min. Centrifuged
8	3·33	3·52	0·90	4·42	+33	Finely divided for 10 min. Centrifuged

When brain is ground in eserine-saline and then extracted at once, without incubation, there usually results an increase of 25–33% in the acetylcholine yield (Table 3, Exps. 2–8). The increase is accounted for by the release of acetylcholine into the saline and by its concurrent replacement in the tissue particles in which there is no appreciable change. These were extracted separately in the last three experiments of Table 3. On incubation, further release and replacement of acetylcholine takes place, accounting for the greater yield under such conditions.

Temperature

The observations of Mann *et al.* [1939] have been confirmed that the production of acetylcholine by minced tissue is much slower at room temperature than at body temperature. There is, however, an effect of temperature in the opposite direction.

The acetylcholine content of a sample of ground brain maintained in 5 c.c./g. eserine-saline for 90 min. at 18–20° C. increased about 50% only. Since grinding alone would increase the yield of acetylcholine by about 25–33%, the increase during the subsequent 90 min. was only small.

When a suspension of ground brain has been shaken for 1–2 hr. there is some impairment of its synthesis of acetylcholine. This change proceeds more quickly at body than at room temperature; it can most easily be demonstrated when the period with eserine is preceded by one without eserine. In the experiments of Table 4 samples were shaken in 5 c.c./g. saline, first for 90 min.

without eserine at different temperatures, and then incubated with eserine for the same period at 36° C. During the preliminary shaking, any acetylcholine released will be destroyed by the cholinesterase, but that in the particulate matter, as we have seen, will scarcely have changed; so that at the beginning of the second period of incubation the samples can be assumed to contain approximately the same amounts of acetylcholine as were originally present in them. The figures in Table 4 were, therefore, obtained by subtracting from the final acetylcholine, as determined in the samples, 3·2 or 3·7 μg./g. respectively, according to the weight of the animals used, the differences representing the amounts synthesized.

TABLE 4. Effect of preliminary shaking, for 90 min., of ground brain (guinea-pig), at different temperatures, on its subsequent synthesis of acetylcholine on incubation at 36° C. for 90 min.

No. of exp.	Temperature in ° C. during preliminary shaking	Acetylcholine in μg./g. synthesized on subsequent incubation
1	− 2	4·39
2	+ 2	9·1
3	+18	4·85
4	+36	3·06

A sample kept at 2° C. for 90 min. did not show any impairment of synthesis on subsequent incubation. The 9·1 μg./g. of acetylcholine synthesized during incubation, after pretreatment at 2° C. (Exp. 2, Table 4), corresponds to the amounts synthesized without such preliminary treatment (compare Table 1). By pretreatment at 18° C. the tissue suffers a definite reduction of its synthetic activity, and this impairment is still more pronounced at 36° C. (Table 4).

Freezing. According to Mann *et al.* [1938] freezing a suspension of minced brain entails complete loss of ability of the suspension to synthesize acetylcholine. In the present experiments it was found that when a suspension of brain material, without eserine, was frozen for 90 min., its ability to synthesize acetylcholine was reduced (Exp. 1, Table 4), but the impairment was not greater than in a sample kept at room temperature. There was, however, a greater impairment, though not a complete loss, when the tissue was frozen for 24 hr. or longer (Table 5). In the first two experiments shown in Table 5 the fresh brain was ground in eserine-saline, and the suspension was frozen; in the last two experiments the intact half-brains were frozen for 3 days. Then one half, while still frozen, was extracted with HCl containing eserine; the

TABLE 5. Synthesis of acetylcholine of brain tissue previously frozen

	Acetylcholine of frozen brain in μg./g.		
Duration of freezing (days)	Control	Incubated for 2 hr. at 36° C.	Amounts of acetylcholine synthesized in μg./g.
1	3·15	4·92	1·77
3	2·11	3·16	1·06
3	1·50	4·76	3·26
3	1·33	(1·67) 2·87	1·54
Average	2·02	3·93	1·91

other half was ground and incubated in eserine-saline at 36° C. for 2 hr. Owing to the rather remarkable loss of acetylcholine from the otherwise uninjured brain while frozen, as shown by the control half, synthesis started from a low level.

In the last experiment the acetylcholine of the particulate matter was determined separately and is given in brackets. The tissue particles were presumably no longer able to build up their normal acetylcholine complement, which would have been of the order of 3·7 μg./g. It is interesting that the value for the particulate matter approximates to that of the control half-brain. This impairment of the tissue's power to build up a normal store of acetylcholine is associated with a reduction in the amount released.

Volume of saline used for incubation

Mann *et al.* [1939] have concluded that when small amounts of tissue are incubated in a large volume of saline, the tissue substances required for synthesis are diluted below the optimum level. This conclusion was confirmed. The effect was best shown, when two equivalent samples were incubated for 90 min. at 36° C., but in different volumes. The half-brains of two small guinea-pigs were distributed in the usual way between the two samples. One was incubated with 5 c.c./g., the other with 20 c.c./g. of eserine-saline. The yield from the former was 11·5μg./g., that from the latter 9·5μg./g. The brains can be taken to have contained initially about 3·2μg./g. and about another 0·8μg./g. was probably synthesized during grinding. Thus, during incubation, about 7·5 and 5·5μg./g. respectively were synthesized.

An even greater depression occurs when a suspension of ground brain is centrifuged, and the supernatant fluid is replaced by fresh solution before incubation. Two samples were suspended in 5 c.c./g. eserine-saline and centrifuged. The one residue was resuspended in its own supernatant fluid and incubated; it yielded 10·58μg./g. The supernatant fluid of the other sample was replaced by an equal volume of fresh eserine-saline; after incubation the yield was 7·41μg./g. The supernatant fluid, incubated separately, yielded 0·65μg./g. This value is lower than the average amount synthesized during the grinding, and shows that no synthesis had occurred in this fluid during incubation. This result confirms the observation of Mann *et al.* that synthesis of acetylcholine does not occur in solution, but needs insoluble brain material, i.e. cell debris or intact cells. Deducting from both yields the estimated original content of 3·7 μg./g. and adding 0·65μg./g. to that for the tissue resuspended in eserine-saline, an estimated synthesis of about 6·9 and 4·4μg./g. occurred in the two cases.

The substances necessary for optimal synthesis in a suspension of ground brain are not derived from the traces of blood present in the brain. Synthesis is not depressed when all traces of blood have been washed out of the brain by a preliminary perfusion of the head from the aorta for 10 min. with eserine-saline. In such an experiment, for which again two brains were used, the control halves extracted immediately yielded 3·34μg./g.; those incubated for 90 min. in 5 c.c./g. eserine-saline yielded 11·14μg./g., giving an increase of 232%.

Table 6. Accelerating effect of glucose on synthesis of acetylcholine in an eserine-saline suspension of ground half-brains incubated for 90 min. at 36° C.

No. of exp.	Total acetylcholine yield in μg./g.		Acetylcholine in μg./g. synthesized during incubation		% increase due to glucose
	Without glucose	With glucose	Without glucose	With glucose	
1	8·5	10·9	4·5	6·9	53
2	9·9	11·2	5·9	7·2	22
3	7·9	11·9	4·7	8·7	85
4	6·6	9·2	3·4	6·0	76

Glucose

Mann *et al.* [1938, 1939] have found that glucose accelerates synthesis of acetylcholine in fresh brain tissue whether sliced or minced. They attributed the failure of Stedman & Stedman [1939] to observe this effect to the fact that these authors had not washed the tissue free from its 'metabolites', and had incubated relatively large amounts of tissue with small amounts of saline, thus causing a correspondingly small dilution of the natural glucose and other factors required for synthesis.

The accelerating effect of glucose has been confirmed, but it has also been observed that if the extractives in a suspension of ground brain have not been sufficiently diluted, or removed by washing, the addition of glucose to the suspension may even exert an inhibitory effect.

Augmentation. For each experiment of Table 6 the brains of two small guinea-pigs were used, halved and distributed in the usual way. In Exps. 1 and 2 the brains were ground in eserine-saline and incubated in 21 c.c./g. to dilute the extractives, 1 mg./c.c. glucose being added to one suspension. The amounts of acetylcholine synthesized during incubation (third and fourth columns) were obtained by subtracting 4μg./g. from the acetylcholine yield of the samples. The 4μg./g. represents the estimated initial acetylcholine content (3·2μg./g.) plus 25% for synthesis during grinding.

In Exps. 3 and 4 the extractives were removed by spinning the saline suspensions (20 c.c./g.) of ground brain and replacing the supernatant fluid with fresh eserine-saline. This was done once in Exp. 3 and twice in Exp. 4. Each residue was taken up with about 5 c.c./g. eserine-saline, with glucose (1 mg./c.c.) in one case, and incubated. The amounts synthesized during incubation were calculated by subtracting 3·2μg./g. from the acetylcholine yields obtained. The yield of acetylcholine in the sample of Exp. 4, containing glucose, was somewhat low; the repeated exchange of supernatant fluid may have deprived the tissue of factors required for synthesis other than glucose, a possibility also envisaged by Mann *et al.*

Inhibition. The results are given in Table 7. Again for each experiment the brains of two small guinea-pigs were used. The samples were ground in eserine-saline and incubated in 5 c.c./g. The samples to which, under these conditions, glucose had been added, yielded less acetylcholine than those without added glucose. The amounts synthesized were obtained by subtracting 4μg./g. from the acetylcholine yield. Inhibition was evident with 1 mg./c.c. glucose, and increased progressively with stronger concentrations.

TABLE 7. Inhibiting effect of glucose on synthesis of acetylcholine in an eserine-saline suspension of ground half-brains incubated for 90 min. at 36–37° C.

Total acetylcholine yield in μg./g.		Acetylcholine in μg./g. synthesized during incubation			
Without glucose	With glucose	Without glucose	With glucose	Glucose in mg./c.c.	Inhibition by glucose in %
10·38	9·22	6·38	5·22	1	18
12·20	10·40	8·20	6·40	1	22
12·00	10·95	8·00	6·95	2	13
11·67	8·87	7·67	4·87	2·2	37
12·01	8·67	8·01	4·67	4	42
11·90	7·94	7·90	3·94	4·1	50
9·22	4·76	5·22	0·76	8	85

The factor of dilution would thus appear to have been a principal cause, at least, of the apparent discrepancy between the findings of the Stedmans and those of Mann *et al.*

Iodoacetic acid

An intracardiac injection, into a guinea-pig, of 70 mg./kg. iodoacetic acid proved lethal within 15 min. Synthesis of acetylcholine in the ground brain, removed immediately after death, appeared to be normal. When, however, strong concentrations of iodoacetic acid, carefully neutralized with NaOH, were added to the medium used for incubation, synthesis was inhibited, even if no intracardiac injection had been given during life. The inhibiting effect of different concentrations of iodoacetic acid is seen when the results given in Table 8 are compared with those given in Table 1. When the brain, instead of

TABLE 8. Effect of iodoacetic acid on synthesis of acetylcholine in a saline suspension

		Acetylcholine in μg./g.			
No. of exp.	Concentration of iodoacetic acid	Control, half-brain	Incubated, half-brain	Difference in %	Notes
1	1 : 1200	3·64	3·50	− 4	
2	1 : 1400	2·92	2·50	−14	
3	1 : 1800	2·77	2·86	+ 3	
4	1 : 1800	2·98	3·34	+12	Sodium pyruvate added
5	1 : 2000	3·43	3·13	− 9	Sodium lactate added
6	1 : 2900	2·96	3·08	+ 4	
7	1 : 4000	2·85	3·75	+32	Sodium pyruvate added
8	1 : 4000	2·87	4·34	+51	
9	1 : 2200	3·53	6·66	+88	Brain not ground but finely divided for 10 min.
10	1 : 2200	4·48	7·28	+63	

being ground, was finely divided with scissors, the effect of iodoacetic acid on incubation was less pronounced (Table 8, Exps. 9 and 10).

The effect of iodoacetic acid was not prevented by adding sodium lactate or pyruvate to the medium (Table 8, Exps. 4, 5 and 7).

Ether

In the absence of eserine. It has been shown that the tissue particles of ground brain incubated in saline, without eserine, retain their acetylcholine. This is not the case when ether is added to the medium. Under these conditions, the acetylcholine of the tissue particles diminishes progressively. The results in Table 9 were obtained with brains of small guinea-pigs ground with 6 c.c./g. ether and 4–5 c.c./g. saline. At higher temperatures (36° C.) the tissue lost its acetylcholine more quickly.

TABLE 9. Acetylcholine content of ground half-brains (guinea-pigs) shaken in an ether-saline mixture at 16° C. for different periods

Time of shaking in min.	0	2	10	20	90
Acetylcholine in μg./g.	3·2	2·3	1·9	1·4	0·6

There are two possible explanations of this effect. The acetylcholine may be released (and then destroyed) from the tissue more quickly than it can be replaced by synthesis, or the ether may change the condition in which the acetylcholine is held in the tissue, so that, without being released, it loses its protection against the cholinesterase. Experiments with dried brain powder (see p. 387) favour the latter explanation.

In the presence of eserine. The observations of Stedman & Stedman [1937, 1939] and of Mann *et al.* [1939], that a suspension of ground brain allowed to stand with eserine-saline and ether at room temperature synthesizes acetylcholine have been confirmed. Grinding the brain for 2 min. with eserine-saline and ether did not immediately increase the acetylcholine yield; when the volume of saline used was less than 2 c.c./g. there was actually a slight loss.

Several factors were found to influence the synthesis, such as the procedure of grinding and the volume of saline used. When samples were shaken for 90 min. at room temperature, the acetylcholine yield varied according to whether the brain had been ground in saline with both eserine and ether present, or whether the eserine or the ether had been added after the grinding. Under either of the last conditions the acetylcholine was less. In the following experiments, therefore, ether and eserine-saline (usually about 2 c.c./g. of each) were used for grinding the brain, and the samples were then made up to the final volumes. The effect of varying the volumes of ether and eserine-saline in the final mixture is shown by the experiments of Table 10. Each value is from a half-brain and has been obtained by subtracting, from the final content observed, either 3·7 or 3·2μg./g. according to the weight of the animal. It will be noted that, in the presence of ether, the optimal value may be as high as 12µg./g. which is higher than the best yield obtained with eserine-saline alone.

TABLE 10. Acetylcholine in µg./g. synthesized by ground half-brains (guinea-pig), in different mixtures of eserine-saline and ether during 90 min. at 16° C.

Eserine-saline (c.c./g.)	ether (c.c./g.) 0	0·5	2	5	10	20	40
20	—	—	0·7	—	0	0·2	0
10	—	—	2·6	—	0·9	1·6	—
5	0·2	—	4·8	5·9	5·3; 5·6	3·8	4·6; 5·1; 3·1
2	0·2	7·6	12·0	8·0	11·1	9·1	6·8
1	—	—	10·3	7·4	8·1	4·8	—
0·08	—	—	10·0	7·8	11·3	6·4	—

A change in volume of eserine-saline from 0·08 to 2 c.c./g. did not clearly influence synthesis in the presence of ether. The 0·08 c.c./g. were needed for dissolving the eserine. With greater volumes of eserine-saline, from 5 c.c./g. upwards, synthesis diminished and, finally, with a volume of 20 c.c./g. it practically ceased. On the other hand, with the exception of a slight depression of synthesis with very large volumes, the volume of ether had no effect. It is interesting to note that an accelerating effect is obtained with relatively small amounts of ether.

The following experiments were carried out to examine the depressing effect exerted by large volumes of eserine-saline in an ether-saline medium. Glucose was added to the eserine-saline; it did not restore synthesis. The following procedure, however, proved effective. The brains of three guinea-pigs were ground in 5 c.c./g. saline, without eserine, the suspension was centrifuged and the supernatant fluid removed. This fluid, when shaken with eserine and ether but without the residue, did not synthesize acetylcholine; if, however, it was used instead of clean saline for preparing the ether-eserine-saline medium in which the brain tissue was shaken, the inhibition otherwise exerted by a large volume of saline was no longer observed. The effects of glucose and supernatant fluid are shown in Table 11. The values are again from separate half-brains and were obtained by subtracting either 3·7 or 3·2µg./g. from the acetylcholine yield.

TABLE 11. Synthesis of acetylcholine by ground half-brains (guinea-pigs) in different media during 90 min. at 16° C.

No. of sample	Content of sample	Acetylcholine in µg./g. synthesized
1	Half-brain. 5 c.c./g. eserine-saline and 10 c.c./g. ether	5·6
2	Half-brain. 15 c.c./g. eserine-saline and 15 c.c./g. ether	0·3
3	Half-brain. 15 c.c./g. eserine-saline plus 1·5 mg./c.c. glucose and 15 c.c./g. ether	0
4	Half-brain. 5 c.c./g. eserine-saline plus 15 c.c./g. supernatant fluid (obtained from 3 other brains, each ground with 5 c.c./g. saline) and 15 c.c./g. ether	4·8
5	Equal volumes of eserinized supernatant fluid and ether	0

It was not possible to determine the acetylcholine in solution, separately from that held in the tissue. The ether forms a more or less homogeneous emulsion with the brain material and the saline, from which the brain material cannot be separated. When the volume of eserine-saline is large, some cloudy fluid may separate out at the bottom of the flask on standing, but, even then, the layer containing the brain material contains a great bulk of aqueous solution. The results obtained when ground brain is shaken in ether, with practically no additional saline, are of interest. The total yield of acetylcholine in the experiments given in the bottom row of Table 10 was between 9·6 (6·4+3·2) μg./g. and 15 (11·3+3·7) μg./g. Since acetylcholine is insoluble in ether, the amounts synthesized may be held in the tissue particles, or in the tissue fluid, or may be orientated at the ether-water interface.

In confirmation of Mann *et al.* [1939] it has been found that synthesis was greatly reduced and sometimes nearly abolished by incubation in eserine-saline with ether at 36° C. instead of at 16° C. The effect of temperature when ether is present has been examined in detail with dried brain (see p. 388).

TABLE 12. Accelerating effect of glucose on synthesis of acetylcholine. Suspension of ground brain in an eserine-saline-ether medium at 16–17° C.

Total yield of acetylcholine in μg./g.		Acetylcholine in μg./g. synthesized in 90 min.	
Without glucose	With glucose	Without glucose	With glucose
5·4	6·3	1·7	2·6
5·8	6·6	2·1	2·9
6·3	8·8	2·6	5·1
4·8	6·0	1·6	2·8

It has been mentioned that the depression of synthesis by dilution in an eserine-saline-ether medium cannot be prevented by glucose. In order to demonstrate an accelerating effect of glucose, conditions had to be chosen which allowed some water-soluble substances derived from the tissue to be removed, without causing an irreversible loss of synthesis. The following procedure proved successful. The half-brains were ground in saline, the suspensions centrifuged and the supernatant fluids removed. Eserine was added to the residues, which were ground for another 30 sec. with 2 c.c./g. ether; another 8 c.c./g. ether and 3 c.c./g. eserine-saline were then added to each sample, and to one 7 mg./g. glucose. The results are given in Table 12. Repeated centrifuging and replacement of the supernatant fluid resulted in more or less complete loss of synthesis, which could not be restored appreciably by glucose.

No inhibiting action of glucose in a medium of eserine-saline with ether has been found in these experiments.

II. FORMATION OF ACETYLCHOLINE BY DRIED TISSUE OF THE CENTRAL NERVOUS SYSTEM

Dried brain powder, although containing only negligible amounts of acetylcholine, retains the ability to synthesize it. Indeed, under the conditions most favourable to its action, it synthesizes acetylcholine much more rapidly and to a much higher maximum yield than the corresponding amount of fresh brain, under comparable conditions. The amounts formed vary with the species, the treatment of the brain before and after drying and with the temperature, medium and duration of incubation.

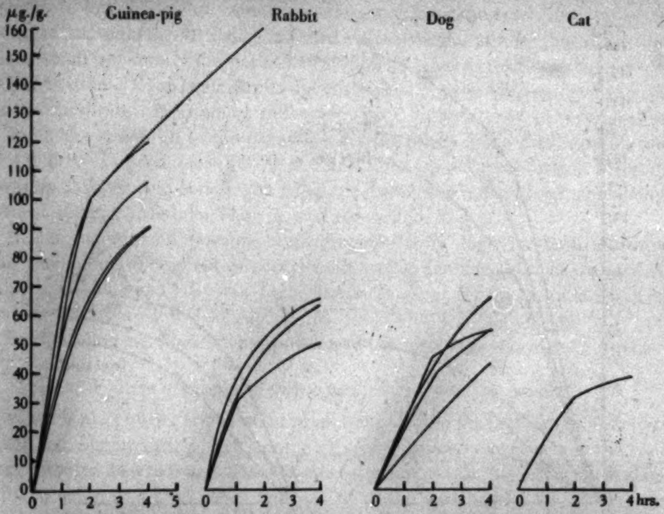

Fig. 2. Synthesis of acetylcholine by brain powder from different species at 18° C. in eserine-saline with ether. Ordinates: acetylcholine in μg./g. Abscissae: time in hours.

The results obtained when 200–250 mg. of brain powder from different species are shaken at 18° C. in about 3 c.c. eserine-saline with 10 c.c. ether are shown in Fig. 2. In each experiment, a number of samples of the same powder were shaken for different periods, and each curve represents the results of one experiment. The acetylcholine formed is expressed in μg./g. of powder, and is plotted as ordinates against the time in hours as abscissae. Of the four species examined, dried brain from the guinea-pig yielded the most active powder, that of the cat the least active one. The amounts of acetylcholine formed during 4 hr. incubation varied between 100 and 120μg./g. for the guinea-pig, and between 40 and 70μg./g. for the rabbit and dog. With rabbit's brain the formation was quicker during the first hour and slower during the later hours than with dog's brain.

Powder of guinea-pig's brain was made by drying six brains, of rabbit's brain by drying three or four brains together. Individual variations were thus partly eliminated. One powder of dog's brain gave relatively low values for synthesis. In this case the cerebellum and medulla had also been removed for drying. All parts were dried separately in the same desiccator, but the amount of P_2O_5 used was apparently insufficient for quick drying of this quantity of tissue, and this might explain the low values obtained.

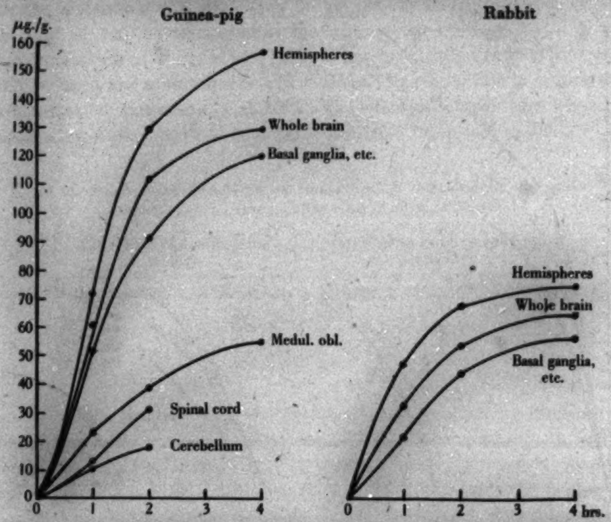

Fig. 3. Synthesis of acetylcholine by dried tissue of different parts of the central nervous system, kept in eserine-saline with ether at 18° C. Ordinates: acetylcholine in μg./g. Abscissae: time in hours.

When the dried powder was kept in the cold over P_2O_5 or $CaCl_2$ its ability to synthesize acetylcholine diminished gradually. Usually there was no diminution during the first week. During the first 3 weeks some powders lost 10%, others up to 30% of their activity. In powders (dog's brain) kept for 3–4 months, 40–50% of the original activity was still retained.

In the early experiments, the brains were often kept frozen for a few days before being dried. The powders obtained from these were about half as active as those from freshly dried brains.

Different parts of the central nervous system. The results are shown in Fig. 3. The samples were treated in the same way as in the experiments of Fig. 2. Powder prepared from the cortex was more active than that from the whole brain, or from any other part separately tested. The brains of four rabbits were divided in the middle line, the right halves of two and the left halves of the other two being dried together. From the four other halves the hemispheres were removed and dried separately from the rest. The activities of the three powders are shown in Fig. 3. In a corresponding experiment, with six guinea-pigs, the medulla, spinal cord and cerebellum were separately dried and assayed (Fig. 3). Dried medulla and spinal cord were less active than dried cerebrum, but the lowest values were obtained from the cerebellum (18μg./g. during 2 hr. shaking). Dried spinal cord did not form a powder, but a wax-like mass which apparently did not take up water or ether as readily as the powder from the brain. Even the medulla did not yield a powder with such a fine grain as the forebrain. Mechanical differences may be concerned in irregularities which make it difficult to compare the activities of the spinal cord, medulla and cerebellum. For instance, during the first hour, spinal cord formed 13μg./g., medulla 23μg./g. and cerebellum 10μg./g.; during the second hour, spinal cord formed 19μg./g., medulla 14μg./g. and cerebellum 8μg./g.

In one experiment, powders were prepared from dog's forebrain, medulla and cerebellum. The yield of acetylcholine after shaking for 2 hr. in eserine-saline with ether at 18° C. was, for the brain 24μg./g., for the medulla 9·5μg./g. and for the cerebellum 1·7μg./g.

In the following experiments powder of the forebrain was always used.

Volume of ether and saline used for treatment of powder

When brain powder is treated at room temperature with different volumes of ether and eserine-saline the amounts of acetylcholine formed in a given time vary greatly. It was found: (1) that no acetylcholine was formed in ether alone (99·5% ether was used, since 0·05 c.c. of water were needed to dissolve the eserine; (2) that acetylcholine was formed in eserine-saline in the absence of ether; but (3) that ether accelerated this formation; and (4) that the volume of eserine-saline greatly influenced the formation of acetylcholine, whereas the volume of ether, once sufficient, did not further influence the result; with optimal volumes the addition of ether might effect a four- to fivefold increase in the yield.

Brain powder, shaken in eserine-saline with ether, takes up both water and ether. The uptake of ether is seen when the dried brain powder is mixed with eserine-saline and ether in a wide test-tube, and the sample is left unstirred, or only occasionally shaken. The brain particles, which at the beginning cover the bottom of the tube, gradually rise to the top of the aqueous phase. Usually samples composed of 300 mg. powder, 3 c.c. eserine-saline and 10 c.c. ether were kept at 18° C. Within 20 min. the finer particles of the powder accumulated at the top of the aqueous phase, the larger ones still lying at the bottom; but eventually these also rose to the top. Thus, a layer of particulate matter was formed which became more compact with time, whilst a deepening layer of relatively clear fluid separated at the bottom. Finally, the sample showed a deep layer of ether at the top and a shallow watery layer at the bottom; between the two the brain material floated, consisting in its upper part of fine particles, giving it a pus-like appearance, and in its lower part of larger reddish particles. After the sample had been stirred, the layers separated out again on standing.

The accelerating effect of ether on the synthesis of acetylcholine is apparent with small amounts of ether, and becomes optimal when about 1 c.c./100 mg. powder are added to a sample, further addition producing no further increase (Table 13).

TABLE 13. Effect of different volumes of ether on synthesis of acetylcholine by dog's brain powder

Each sample contained 300 mg. powder and was shaken for 2 hr. at 18° C. with 4–5 c.c. eserine-saline and the volume of ether indicated.

Ether in c.c.	0	0·3	0·5	1	3	6
Acetylcholine in μg./g.	7·5	13·5	15·3	21·8	25·3	25·1

When the ether is present in a sufficient proportion, the formation of acetylcholine rises to a maximum with increase in the volume of eserine-saline added, and then decreases with further increase (Table 14).

TABLE 14. Effect of different volumes of eserine-saline on synthesis of acetylcholine by dog's brain powder

Each sample contained 300 mg. powder and was shaken for 2 hr. at 18° C. with 5 c.c. ether and the volume of eserine-saline indicated.

Saline in c.c.	0·05	0·2	0·4	0·8	1	2	3	4·5	8
Acetylcholine in μg./g.	0	1·9	4·8	12·7	23·3	31·9	32·0	25·3	14·6

In this experiment with 300 mg. powder, corresponding to about 1·4 g. fresh tissue, the optimal volume of saline was 2–3 c.c. Smaller volumes of saline were apparently insufficient. With larger volumes, a water-soluble factor, necessary for optimal synthesis, apparently becomes too greatly diluted. Optimal conditions for the synthesis of acetylcholine, therefore, were obtained when the volume of eserine-saline was about 2–3 times that of the water present in the fresh tissue. When brain powder was kept in eserine-saline without ether, much larger volumes of saline could be used without impairing the synthesis of acetylcholine.

When, in the following section, reference is made to synthesis in eserine-saline with ether, without stating details, 200–300 mg. powder were mixed with 2–3 c.c. eserine-saline and 10 c.c. ether.

TABLE 15. Synthesis of acetylcholine by guinea-pig's brain powder in different media (for details see text)

No. of sample	Content of sample (in addition to eserine and 10 c.c. of ether)	Acetylcholine in μg./g.
1	200 mg. powder and 3 c.c. saline	62
2	200 mg. powder and 6 c.c. saline	50
3	200 mg. powder and 6 c.c. saline with 1·5 to 3 mg. glucose/c.c.	50
4	200 mg. powder, 3 c.c. saline and 3 c.c. supernatant fluid obtained from 250 mg. powder	77
5	3 c.c. supernatant fluid from 250 mg. powder	2
6	3 c.c. saline and 200 mg. powder (after twice suspending in saline, centrifuging and removing the supernatant fluid)	12

Similar results to those described for dog's dried brain were obtained with guinea-pig's brain powder. For instance, samples of 200 mg. of the latter, kept for 90 min. at 18° C. in 3 c.c. eserine-saline with 10 or 20 c.c. of ether, formed 46·3 and 48·0μg./g. acetylcholine respectively. In another experiment, the medium consisted of 10 c.c. ether with 2·7 and 6·7 c.c. of eserine-saline respectively; the yields of acetylcholine were 53 and 35μg./g. The decrease caused by the large saline volume was not prevented by adding glucose to the saline (sample 3, Table 15). When, however, the increased volume of eserine-saline was replaced by a corresponding volume of supernatant fluid, obtained from another sample of brain powder, there was no longer a decreased but sometimes even an increased acetylcholine formation. In such an experiment, the results of which are given in sample 4, Table 15, about 500 mg. powder were shaken for a few minutes in 6 c.c. saline, without eserine and ether, the suspension was centrifuged and the supernatant fluid was removed and used for making up the sample. This fluid, when shaken without the residue but with eserine and ether, synthesized very small amounts of acetylcholine (sample 5, Table 15).

The importance for synthesis of acetylcholine, of a water-soluble factor extracted from the powder by saline, is further evident from the fact that synthesis was greatly reduced when, after spinning down a saline suspension of brain powder, the residue was washed and resuspended in fresh eserine-saline (see sample 6, Table 15). The soluble factor was not affected by separate incubation of the supernatant fluid, with or without ether, at 18 or 36° C., or indeed even by boiling.

Distribution of the formation of acetylcholine in eserine-saline with ether

Only traces of acetylcholine can be extracted from the ether layer. In order to determine separately the amounts in the eserine-saline and in the brain material, samples of dog's brain powder were kept at 18° C. in a separating funnel and left unstirred during the last few minutes. The eserine-saline separating out at the bottom was collected. The residue remaining in the funnel was washed twice with a few c.c. of eserine-saline, previously shaken with ether. The original eserine-saline and the washings were pooled. In the extraction of acetylcholine the ether and the particulate matter were usually treated together. The results are given in Fig. 4. The upper tracing gives the total acetylcholine yield; the lower that of the particulate matter. When once the latter reached a certain value, between 11·4 and 12 μg./g. in this experiment, it remained constant. Any further increase in the total acetylcholine must have resulted from an increase in the acetylcholine of the eserine-saline. Small amounts of acetylcholine appeared in the eserine-saline before the particulate matter reached its full value. In the experiment illustrated by Fig. 4, the sample taken after 25 min. yielded 7·7 μg./g. from the particulate matter and 2·8 μg./g. from the saline.

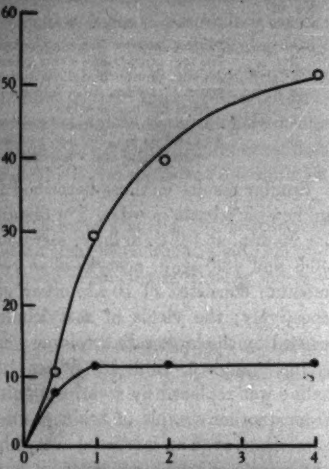

Fig. 4. Distribution of acetylcholine, synthesized at 18° C. in eserine-saline with ether, by dog's brain powder, in the particulate matter (lower curve) and the aqueous layer. Upper curve total acetylcholine. Ordinates: acetylcholine in μg./g. Abscissae: time in hours.

In other experiments, with brains of different species, the acetylcholine content of the particulate matter was determined after incubation for 2 hr. in the same manner and compared with the acetylcholine formation by powder (Table 16). There was a general correspondence. Powder of the guinea-pig's brain showed the greatest acetylcholine formation and the highest value for the particulate matter.

In order to compare the acetylcholine content of the particulate matter from powder with that of fresh brain tissue, the former was calculated in μg. per

TABLE 16. Content of acetylcholine by particulate matter of brain powder suspension compared with fresh brain

Animal	Synthesis of acetylcholine in 2 hr. μg./g. powder	Acetylcholine in particulate matter		Acetylcholine in μg./g. fresh brain
		In μg./g. powder	In μg./220 mg. powder (equiv. 1 g. fresh brain)	
Cat	33	9·5	2·1	1·6
Dog	46	10·6	2·3	1·3
Dog	42	11·4–12·0	2·5–2·6	
Dog	41	14·0	3·1	
Rabbit	44	13·5	3·0	1·6–1·8
Guinea-pig	67–90	20·0	4·4	3·2–3·7
Guinea-pig	64	21·0	4·6	
Guinea-pig	93	23·8	5·0	

220 mg. powder, the approximate equivalent of 1 g. fresh tissue. Although of the same order in both (columns 4 and 5, Table 16), it was in all species higher in the material from powder than in fresh brain. In the case of the guinea-pig, where the difference was from 30 to 50%, a sufficient number of brains have been examined to allow such a comparison. The figures for the fresh brain were derived from forty-five individual determinations, and each powder was prepared from six brains. Possibly the figures for the particulate matter from the powder are a little too high, and include small amounts of acetylcholine in solution, since the separation may not be complete with the method employed. In addition, the particulate matter still includes fluid which may contain acetylcholine, as was found in corresponding experiments with fresh brain. The main result is that powder of dried brain, when kept in eserine-saline with ether, builds up in the tissue particles a store of acetylcholine of the same order as is present in fresh brain. No store is built up unless eserine is present.

In the eserine-saline layer all the acetylcholine is in free and active form. The same acetylcholine equivalents were obtained when one part of the saline layer is assayed directly on the frog's rectus muscle, the other part after extraction with acid and boiling.

The acetylcholine in the eserine-saline layer is not formed there, but is released into it from the particulate matter where it is formed, as shown by the following experiment. Brain powder was kept in a separating funnel with eserine-saline and ether at 18° C. for 3 hr., but after 1½ hr. the liquid at the bottom of the funnel was removed and replaced by fresh eserine-saline. The removed liquid was divided into two parts, one being extracted at once, while the other, after the addition of ether, was kept at 18° C. for another 1½ hr. Both fractions yielded the same acetylcholine equivalent. No acetylcholine, therefore, was formed when the liquid was shaken without brain particles. On the other hand, the fresh eserine-saline after shaking for a further 1½ hr. with the particulate matter was found to contain acetylcholine.

Distribution between powder and eserine-saline in the absence of ether

Dried brain, kept in eserine-saline without ether, is unable to build up a store of acetylcholine in the tissue particles. The value eventually reached was less than 1 μg./g. It has not been possible to determine it exactly, since, on centrifuging a sample, the particulate matter forms a rather bulky slime, containing a relatively large proportion of fluid which cannot be separated. The concentration of acetylcholine in the slime was found to depend on that in the saline. For instance, two samples of 200 mg. of powder of guinea-pig's brain were kept for 2 hr. at 19° C. in 9 c.c. eserine-saline; another 9 c.c. were added to one sample, and both were kept for a further 10 min. They were then centrifuged, and the supernatant fluids were poured off. In each case, the slime weighed 2·5 g. and contained therefore about 2·3 c.c. fluid. The slimes from the 9 and the 18 c.c. sample yielded 3 and 1·5μg./g. acetylcholine respectively; the supernatant fluids contained 13·5 and 14μg./g., which were distributed in 6·7 and 15·7 c.c. of fluid. The concentrations in the fluids, therefore, were 2 and just under 1μg./c.c., those in the corresponding slimes being 1·2 and 0·6μg./g. respectively.

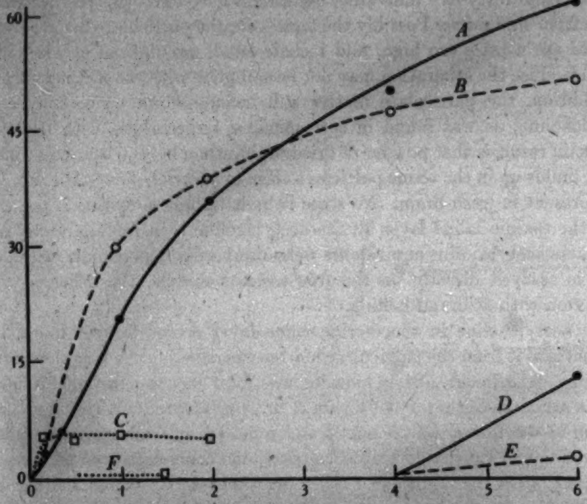

Fig. 5. Effect of temperature on synthesis of acetylcholine by dog's brain powder kept in eserine-saline with ether. A and D at 17° C.; B and E at 25° C.; C and F at 36·5° C. The eserine was added to samples D and E after 4 hr., to sample F after 30 min. Ordinates: acetylcholine in μg./g. Abscissae: time in hours.

Temperature

Eserine-saline with ether. Samples were shaken at temperatures between 3 and 37° C. Within this range, an increase in temperature had a twofold effect. The initial rate of formation of acetylcholine was increased, but during incubation the synthetic power of the brain material declined at a rate which also increased with the temperature (Fig. 5). This decrease was not due to the formed acetylcholine. One sample (D) was kept at 17° C. and another (E) at 25° C. for 4 hr. without eserine, so that no acetylcholine could accumulate; eserine was then added and the samples were kept for another 2 hr. at these temperatures. The samples yielded 12·5 and 2μg./g. acetylcholine respectively, i.e. practically the same quantities as were formed during the third 2 hr. period in the experiments shown in (A) and (B). A sample kept at 17° C., without eserine for 24 hr. and then with eserine for another 2 hr., yielded on extraction less than 0·5μg./g. acetylcholine. This condition was reached within 30 min. at body temperature. There were only traces of acetylcholine (less than 0·5μg./g.) in a sample incubated at 36·5° C., first without eserine for 30 min. and then with eserine for 1 hr. (Fig. 5 F).

In order to demonstrate the increase in the formation of acetylcholine with the increase in temperature, the period of incubation must, therefore, be kept short when temperatures over 30° C. are employed. For instance, with dog's dried brain an incubation for 10 min. yielded 2·4μg./g. at 17° C. and 5·2μg./g. at 36·5° C., although at the latter temperature the rate of synthesis must have fallen by one-half at the end of this time. The amounts synthesized in 1 hr. at different temperatures are shown in Table 17. Similar results were obtained with powder of guinea-pig's brain.

TABLE 17. Synthesis of acetylcholine in 1 hr. at different temperatures by dog's brain powder suspended in a mixture of eserine-saline and ether

Temperature in ° C.	5	10	17	25	30	36·5
Acetylcholine in μg./g.	7·6	11·9	20·6	29·6	17·9	5·2

The question whether the inactivation is wholly an effect on the synthetic mechanism of the particulate matter, or partly on some factor contained in the watery phase of the brain-ether-saline system, has not yet been settled by the experiments so far completed. Material inactivated by preliminary incubation at 36° C. partly regains its synthetic power on addition of fresh supernatant fluid obtained from a saline suspension of brain powder. For instance, a sample of dried guinea-pig's brain shaken for 2 hr. in eserine-saline with ether at 17° C. had formed 75μg./g. acetylcholine, but only 1·7μg./g. when preceded by incubation for 30 min. at 36° C. without eserine. When, however, the saline was exchanged, after the preliminary incubation, with supernatant fluid from a saline suspension of brain, then the value of 1·7μg./g. rose to 21μg./g. On the other hand, it has been mentioned earlier that the soluble factors contained in the watery phase are not affected by separate incubation of supernatant fluid with ether at 18 or 36° C., or even by boiling.

The fact that there is no appreciable loss of acetylcholine already formed, as a result of the incubation, or in the process of extraction, is clear from the following experiment. After the samples had been inactivated at 36° C. in ether-saline, eserine and 100μg. of acetylcholine were added, and incubation was continued for another hour either at 17 or at 36° C. The samples then yielded on extraction 100 and 95μg. of acetylcholine respectively.

Eserine-saline without ether. Brain powder at room temperature in eserine-saline alone forms acetylcholine, but less than when ether is also present. This difference for dog's brain powder is shown in Fig. 6. At 36° C. the amounts of acetylcholine formed were somewhat greater. With powder from the guinea-pig's brain, the acetylcholine yield usually differed only slightly, or not at all, when the samples were shaken at 36° C., instead of at 18° C. The amounts formed without ether by guinea-pig's brain during 2 hr. were between 16 and 25μg./g., as compared with 60–100μg./g. formed with ether in the system. Powder of the rabbit's brain, although less active than that of the guinea-pig when shaken in eserine-saline with ether, was as active or even more active than that of the guinea-pig when in eserine-saline alone. With one powder 40μg./g. were formed during 2 hr., but usually the amount was about 20μg./g.

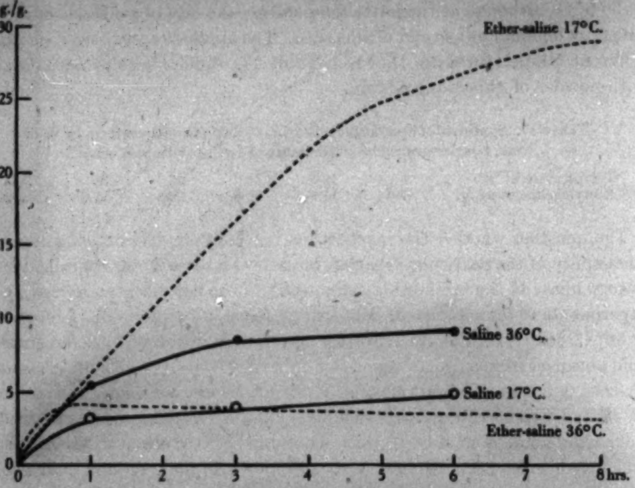

Fig. 6. Effect of temperature on synthesis of acetylcholine by dog's brain powder in eserine-saline (continuous line), compared with treatment in eserine-saline with ether (dotted line). Ordinates: acetylcholine in μg./g. Abscissae: time in hours.

Preliminary incubation in saline without eserine had a similar inactivating effect to that produced by ether-saline. At 36° C. the inactivation of the powder proceeded more quickly than at room temperature. This is best shown as follows. A sample of guinea-pig's brain powder kept for 2 hr. at 20° C. in eserine-saline with ether yielded 90μg./g. acetylcholine. This yield was reduced to (*a*) 56μg./g. and (*b*) 27μg./g. by previous immersion for 30 min. in saline, without eserine and ether, at (*a*) 20° C. and (*b*) 36° C.

Comparison of the inactivation of brain powder at different temperatures in ether, in ether-saline and in saline. No detectable inactivation was observed with powder which had been kept at 20° C. in ether alone for several hours; when kept for 24 hr. under these conditions, subsequent synthesis in eserine-saline with ether was decreased by 40–50%. In the experiment shown in Table 18, samples of 200 mg. dried guinea-pig's brain were kept for 30 min., without eserine, either at 20 or at 36° C. in the media indicated in the table; the samples were then shaken for 2 hr. at 20° C. in eserine-saline with ether. The powder yielded 90μg./g. when shaken for 2 hr. in this medium without previous treatment, and it will be seen that the preliminary treatment in ether at either temperature had no important effect on this yield, whereas treatment in ether-saline caused slight inactivation at 20° C., and very marked inactivation at 36° C. Saline alone caused more inactivation than ether-saline at 20° C., but at 36° C. it caused less.

TABLE 18. Inactivation of brain powder at different temperatures in ether, ether-saline and saline

Temperature during preliminary incubation ° C.	Acetylcholine yield in μg./g. per g. powder of guinea-pig's brain, kept at 20° C. in eserine-saline with ether for 2 hr. following preliminary incubation for 30 min. in		
	Ether	Ether-saline	Saline
20	88	74	53
36	80	3	26

Effect on cholinesterase activity. The presence of ether in the saline greatly inhibits the activity of the cholinesterase of powdered brain; but there is no further or progressive loss of the activity of this enzyme with incubation for several hours in ether-saline at 17° C. and a slight loss only with incubation at 36° C.

TABLE 19. Effect of calcium on synthesis of acetylcholine with powdered brain

No.	Animal	Medium	Acetylcholine in μg./g. (a) Control	Acetylcholine in μg./g. (b) Increased $CaCl_2$	$CaCl_2$ mg./c.c. saline in (b)
1	Dog	Eserine-saline with ether	43	6	3·5
2	Dog		24	7	3·5
3	Dog		24	11	1
4	Guinea-pig		34	8	3·5
5	Rabbit	Eserine-saline	19	Less than 1	4
6	Guinea-pig		20	3	4

Calcium chloride

Mann *et al.* [1939] found that $CaCl_2$ greatly diminished the formation of acetylcholine by fresh brain tissue. The effect was explained, in the main, by a change in cell permeability preventing the release of acetylcholine.

The results of the present experiments are given in Table 19. The extra calcium was found to be precipitated by the phosphate normally used for buffering the saline. In these experiments, therefore, the phosphate buffer was omitted. This by itself entailed a decrease in the final yield of acetylcholine of about 10–20%. Samples of 300 mg. powdered brain were shaken for 1½–2 hr. at 18° C. The calcium not only prevented the release of the formed acetylcholine, but also reduced synthesis in the particulate matter. The 8μg./g. of acetylcholine, synthesized in Exp. 4, were extracted from the particulate matter; the liquid contained no acetylcholine. The particulate matter of the control sample yielded 20μg./g.

Potassium chloride

Mann *et al.* [1939] found that potassium increased the yield of acetylcholine formed by respiring tissue slices. Since the acetylcholine held in the tissue slices decreased under this condition, the potassium was thought to catalyse the release of acetylcholine. The following results may be compared.

Eserine-saline with ether. A high concentration of potassium did not influence the synthesis of acetylcholine. There was also no lowering of the concentration of acetylcholine in the particulate matter. A sample of guinea-pig's brain powder was kept at 20° C. for 1½ hr. with 5 mg. KCl/c.c. saline. The particulate matter yielded 22·7μg./g., as compared with 20–23·8μg./g. without increased potassium concentration (see Table 16).

Table 20. Effect of KCl on formation of acetylcholine by brain powder shaken in eserine-saline

Animal	Acetylcholine in μg./g.		Concentration of KCl in mg./c.c.	Period of shaking in min.
	Control sample	KCl sample		
Guinea-pig	16	20	2·5	30
Rabbit	25	40	2·9	120
Guinea-pig	15	20	3·3	30
Guinea-pig	17	26	3·3	90
Guinea-pig	25	33	3·3	120
Guinea-pig	20	31	4·0	60
Guinea-pig	19	34	4·0	120
Guinea-pig	20	30	4·0	60
Guinea-pig	33	59	4·0	150
Guinea-pig	27	33	4·0	120

Eserine-saline. A high potassium concentration increased the amount of acetylcholine formed by brain powder (Table 20). It was not possible to determine its effect on the acetylcholine content of the particulate matter, as the amounts of acetylcholine actually held in the tissue particles were too small to be determined accurately (see p. 387).

Preliminary experiments on the role of atmospheric oxygen

Oxygen uptake of brain powder in saline at 20° C. One experiment was carried out. Two samples of 200 mg. each of guinea-pig's brain powder were introduced into the side-bulbs of ordinary Warburg manometer vessels. The main vessel contained eserine-saline, the KCl concentration of which had been increased to 4 mg./c.c. The inner compartment contained KOH for CO_2 absorption. After equilibrium had been established, the powder was tipped into the main vessel. This resulted in the development of 19 and 21 cu.mm. gas respectively within 1–2 min. The positive pressure produced by the gas, which was obviously liberated from the dry powder, disappeared entirely in an hour in one sample and practically entirely in the other. After that there was no further change in the manometer during the second hour, and the final readings, after shaking for 2 hr., showed a gaseous change of −4 and +9 cu.mm. respectively. If the total negative pressure (i.e. −23 and −12 cu.mm. respectively) occurring after the positive pressure had been developed is interpreted as oxygen uptake, it would correspond to 115 and 60 cu.mm. O_2/g. powder. This would be an extremely small oxygen uptake. On extraction, each sample yielded 36 μg./g. acetylcholine. More than 80% of this amount must have been formed during the first hour while the negative pressure developed.

Synthesis in eserine-saline with ether at 20° C. *under anaerobic conditions.* When the vessels used for incubation were filled with nitrogen instead of air, synthesis proceeded from the first at a lower rate than in the presence of oxygen, and eventually came to a standstill. This stoppage may have resulted from the accumulation under anaerobic conditions of a metabolic product which prevented further synthesis, or the synthesis may have initially depended on the presence of traces of oxygen, so that the process stopped when these were used up.

TABLE 21. Synthesis of acetylcholine in eserine-saline and ether under anaerobic conditions

No. of exp.	Animal's brain	Period of incubation in min.	Amounts of acetylcholine in μg./g. formed in		Passage of N_2 in min.	Notes
			Air	Nitrogen		
1	Dog	90	33·3	23·3	3	Incubated in Krebs bottles
2	Dog	120	40·7	23·7	3	
3	Dog	240	55·5	24·5	3	
4	Dog	120	40·0	10·0	4	Incubated in Dixon-Keilin flasks. Preliminary incubation for 1½–2 hr. in ether to allow oxygen absorption
5	Dog	100	16·9	7·5	4	
6	Dog	150	25·5	3·0	3	
7	Dog	120	30·0	8·6	4	
8	Guinea-pig	120	93·4	50·0	3	
9	Guinea-pig	120	71·0	58·0	15	
10	Guinea-pig	120	38·1	40·6	15	

The first three experiments of Table 21 were done by shaking the samples in Krebs bottles. Ether and eserine-saline were first introduced into the bottle and nitrogen was bubbled through the solution for several minutes before the powder was added. The lids were then placed on the bottles and nitrogen passed through the vessels for another 3 min. when the taps were closed. Control samples were treated in the same way, but, after the nitrogen had been passed through the vessels, aerobic conditions were again restored. The amounts of acetylcholine formed in nitrogen were practically identical in all three experiments, whereas the amounts synthesized in air increased with prolonged incubation. This indicated that the synthesis in nitrogen had come to a standstill during the first 90 min., and that, with shorter periods of incubation, the difference between the amounts formed in nitrogen and air might be smaller. Evidence for this assumption is illustrated in Fig. 7.

When synthesis in nitrogen has come to a standstill, the powder has not lost its ability to form acetylcholine. If air was admitted at that moment, i.e. after about 90 min., synthesis again set in and proceeded at the same rate as in the control sample. In the experiment shown in Fig. 7, one sample was first kept for 2 hr. in nitrogen and then for another 2 hr. in air. The result is given by the continuous line, starting from the 2 hr. point.

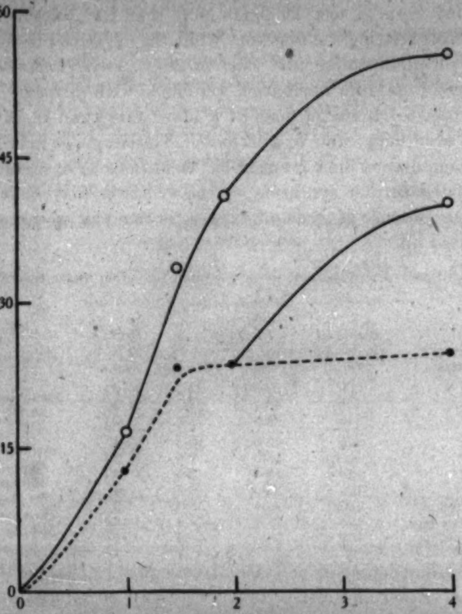

Fig. 7. Synthesis of acetylcholine by powder of guinea-pig's brain in eserine-saline with ether at 20° C. in air (continuous line) and in nitrogen (dotted line). Ordinates: acetylcholine in μg./g. Abscissae: time in hours. For details see text.

In Exps. 4–10, Table 21, the samples were shaken in Dixon-Keilin flasks. This allowed the use of an oxygen absorbent (chromous chloride), which was put into the bottom taps. The powder and the ether were introduced into the main compartment, the eserine-saline into the side-bulb. Nitrogen was passed through the flasks for 3–15 min., when they were closed and the oxygen absorbent admitted. The flasks were shaken in this condition for 1½–2 hr. at 20° C. to allow traces of oxygen, which might still have been present, to be absorbed. No synthesis or inactivation of the powder presumably occurred during this incubation in ether without saline (see p. 391). After the preliminary period, the eserine-saline was poured into the main flasks and synthesis allowed to begin. Control samples were treated in the same way up to the mixture with eserine-saline, but aerobic conditions were then again restored. Although the depression of synthesis in the four experiments with dog's brain powder in Dixon-Keilin vessels is greater than in Krebs vessels, powder of the guinea-pig's brain still synthesized large amounts of acetylcholine even in the former.

TABLE 22. Effect of strong concentrations of NaCN on synthesis of acetylcholine

Brain powder from	Medium	Acetylcholine in μg./g. Control sample	Acetylcholine in μg./g. NaCN sample	Inhibition by NaCN in %	Concentration of NaCN in saline in %
Dog	Eserine-saline with ether	27	28·6	0	0·29
Dog	Eserine-saline with ether	20	18·2	9	0·4
Guinea-pig	Eserine-saline with ether	64	52	19	0·33
Guinea-pig	Eserine-saline	24	26	0	0·02
Guinea-pig	Eserine-saline	24	22	8	0·2
Guinea-pig	Eserine-saline plus 2 mg. KCl/c.c.	56	34	39	0·18
Rabbit	Eserine-saline	40	25	37	0·2

Sodium cyanide

Quastel *et al.* [1936] had observed inhibition of 66–79% of acetylcholine formation, when 0·06% potassium cyanide had been added to the medium incubated with tissue slices of rat's brain.

In the present experiments with brain powder, concentrations of NaCN usually used for inhibition of tissue respiration had no effect on the synthesis of acetylcholine. With much stronger concentrations, slight inhibition was observed, but the effect was not obtained regularly. The results are tabulated in Table 22. When the medium contained ether and saline, the concentration given for NaCN assumes that it is all in the saline. The NaCN was carefully neutralized with HCl before it was added to the samples, which were shaken at 20° C. for 2 hr.

Glucose

No effect of added glucose could be observed when the powdered brain was treated in eserine-saline at 20 or 36° C. with 1–3 mg./c.c. glucose, nor in eserine-saline with ether at 20° C., with 1–10 mg. glucose/c.c. saline. In one experiment, before the incubation in eserine-saline with ether, the powder was taken up in saline and centrifuged, the supernatant fluid being then replaced by eserine-saline. The addition of glucose again had no effect. These negative results with the dried brain powder are in striking contrast to those obtained by Mann *et al.* and to the results obtained in the present experiments with fresh brain material.

Iodoacetic acid

The inhibiting effect is shown in Table 23 with samples of dog's brain powder shaken in eserine-saline with ether for 2 hr. at 18° C.

TABLE 23. Effect of iodoacetic acid on synthesis of acetylcholine

Acetylcholine in μg./g.		Inhibition in %	Concentration of iodoacetic acid in saline in %
Control sample	Iodoacetic acid sample		
24·7	10·9	56	0·067
24·7	2·0	92	0·17
35·0	11·0	69	0·13

DISCUSSION

The results show that synthesis of acetylcholine is not confined to respiring fresh brain tissue, but occurs also with dried brain powder. As the oxygen uptake of such a powder is not more than a small percentage, at most, of that of fresh tissue, it is difficult to assume a direct relationship between synthesis and tissue respiration. The apparent interdependence of the two in fresh tissue incubated in saline, as observed by Mann *et al.*, may be explained differently, when the various factors involved in the formation of acetylcholine are considered.

Mann *et al.* [1938] showed, not only that synthesis occurs in respiring brain tissue, but also that the synthesized acetylcholine appears first in an inactive form in the tissue, bound to some constituent, and that it is then released into the saline. In the early stages of their experiments they were not aware that tissue particles, such as cell debris, retain their acetylcholine. They were, therefore, led to assume the presence in their suspensions of a 'precursor substance'. This interpretation has been contested by Trethewie [1938] and by Stedman & Stedman [1939], who pointed out that the precursor substance of the suspensions was identical with the cell debris. Subsequently, Mann *et al.* [1939] agreed that the bound acetylcholine is in the cell debris, so that there is no longer any disagreement on matters of fact. Mann *et al.* further distinguished substances acting directly on the formation of acetylcholine from those acting indirectly on the mechanism of its release. The results of the experiments here described are in agreement with these conceptions and, in addition, show that brain tissue has a tendency to replace, by synthesis, any acetylcholine which is released. In this way the tissue retains its acetylcholine store at a constant level, though it is apparently unable to increase this level to any appreciable extent. The acetylcholine content of the particulate matter of fresh-ground brain, incubated in saline with or without eserine, remains practically unchanged. The level maintained corresponds to the acetylcholine which can be extracted from the fresh brain tissue. This level may decrease under certain conditions, as in the presence of ether, which appears to deprive the tissue acetylcholine of its protection against the tissue cholinesterase (see later); or when synthesis comes to an end, while release into the medium continues; or, thirdly, when the tissue has partly or wholly lost its ability to synthesize acetylcholine. The last condition exists in brain tissue which has been frozen for some time. A similar condition is observed in peripheral nerves in the early stages of degeneration [Feldberg, 1943].

The assumption that those elements in the tissue in which acetylcholine is formed and deposited in a protected form, maintain their store of it at a constant level, implies that the release of acetylcholine from it must act as a stimulus to renewed synthesis, and that, on the other hand, if synthesis, otherwise initiated, tends to increase the store above the normal, the excess must be automatically released. If the release were inhibited, synthesis would cease too. It is therefore not always easy to distinguish clearly between effects promoting release from those promoting formation. The dependence of the synthesis in fresh brain tissue, suspended in saline, on tissue respiration and body temperature, may be mainly due to the effects of these conditions on the release of acetylcholine, rather than directly on its synthesis. This would explain why, in an ether-saline medium, synthesis occurs also at room temperature and without the normal tissue respiration. Such an explanation is not incompatible with the fact that increase in temperature may accelerate the rate of synthesis, as initially in saline with ether, under conditions where the release is not the limiting factor; nor does it exclude some oxidative metabolism as a condition of synthesis. The experiments with brain powder have, in fact, shown that synthesis soon comes to a standstill under anaerobic conditions. It is not clear whether the initial synthesis in nitrogen was dependent upon traces of oxygen, or whether an anaerobic and an aerobic reaction must be distinguished.

Synthesis of acetylcholine did not take place in the absence of tissue particles, but that does not mean intact cells; cell debris apparently retains this property. In addition, a water-soluble, heat-stable substance of unknown type was necessary for optimal synthesis. It was not glucose; it may be an intermediate required for the synthesis, or a source of energy for the reaction, or a kind of co-enzyme.

With regard to the theory of acetylcholine as transmitter at central synapses, its synthesis by tissue of the central nervous system must be viewed in conjunction with all the other evidence. It is important to recognize that nervous tissue is not the only tissue capable of synthesizing acetylcholine. Human placenta, which is free from nervous tissue, not only contains large amounts of acetylcholine [Chang & Gaddum, 1933] and of cholinesterase [Torda, 1942], but it synthesizes acetylcholine, when ground and incubated in a saline medium containing glucose [Feldberg, 1945]. Viewed in conjunction with the central actions of acetylcholine as well as of eserine, with the presence of a high concentration of a specific cholinesterase [Mendel & Rudney, 1943], with the continuous release of acetylcholine from perfused brain and spinal cord and its increased release from the latter during increased activity, the synthesis forms an additional and important link in the chain of evidence. There is, at present, certainly not sufficient evidence to apply the acetylcholine theory to all central synapses, but it is difficult to discard the theory for the transmission across a number of synapses in the central pathway of autonomic and motor neurons.

Comparison with peripheral nervous system. In comparison with sympathetic ganglia and peripheral cholinergic nerves, brain tissue contains a relatively low concentration of acetylcholine [MacIntosh, 1941]. A low store, but a great ability of replacement, may be characteristic of a tissue which, like the brain, exhibits a continuous release of acetylcholine [Chute, Feldberg & Smyth, 1940]. The conception, however, that the release and replacement of acetylcholine in peripheral cholinergic nerves occurs on excitation only, and is therefore discontinuous, may be incorrect; the difference may be quantitative only. The appearance of large amounts of acetylcholine in the venous effluent from the digestive tract, in the presence of eserine, has been attributed to the continuous release and synthesis of acetylcholine by the nerve plexus present in the wall [Feldberg & Rosenfeld, 1933; Dikshit, 1938]. The cells of this plexus have been regarded, indeed, as automatic centres. But there are other indications of a continuous, although much smaller release of acetylcholine from cholinergic nerves, if we accept the common assumption that the effects of eserine and of similarly acting substances are due to inhibition of cholinesterase activity. The effects of eserine, in reducing the heart rate after the vagi have been cut, may be explained by an action of the parasympathetic ganglia in the heart as weakly automatic centres, causing a continuous release of acetylcholine at the nerve endings, but involving amounts so small as to remain subliminal without eserine. An extension of this idea to nerve structures not including nerve cells might account for the fascicular twitchings and fibrillations produced by eserine in a voluntary muscle, even after recent section of its motor nerve. The fascicular twitchings may be due to the release of acetylcholine caused by random impulses passing down the fibres of the divided nerve [Bacq & Brown, 1937]. For the fibrillary twitchings a different explanation seems to be required. We may picture a more or less continuous release of acetylcholine from the motor nerve endings of the 'resting nerve', so slight as to be eliminated efficiently by the cholinesterase, until the action of this is inhibited by eserine. This explanation would account for the regularity with which the fibrillary twitchings are produced by eserine. Nerve fibres lose their ability to synthesize acetylcholine from 48 to 72 hr. after section, at a time when the conduction of impulses along them is still intact [MacIntosh, 1938]; within the first 24 hr. synthesis of acetylcholine is unimpaired [Feldberg, 1943]. It is therefore in accordance with the above suggestion that eserine causes fascicular twitching and fibrillation, when injected about 24 hr. after nerve section, but not when injected after 48 hr. or later [Kato & Langley, 1914].

Calcium has an antagonistic effect on the twitchings and fibrillations produced by eserine [Kato & Langley, 1914; Loewi, 1922]. This could be explained if it were assumed that calcium inhibits the continuous release and synthesis of acetylcholine at the motor nerve endings, on the analogy of its demonstrated action on brain tissue both fresh and dried. Such quantitative differences as can be observed between the tissues of the central and peripheral nervous systems with regard to the spontaneous release and synthesis of acetylcholine, may, indeed, find their explanation in differences in ionic equilibria. Similar conceptions have been applied to the electrical activity of the nervous system. For instance, when the diffusible calcium content of the tissue fluid is low, a peripheral nerve may exhibit spontaneous activity [Brink & Bronk, 1937], which has been compared with the spontaneous activity of the central nervous system. According to Arvanitaki [1939], the function of peripheral nerves is essentially an oscillatory one which, under normal conditions, is highly damped.

Glucose. The following facts suggest that the accelerating action of glucose is on the synthesis proper and not on the release: (1) a sympathetic ganglion perfused without glucose is unable to restore the acetylcholine released from the tissue on preganglionic stimulation [Kahlson & MacIntosh, 1939]; (2) potassium, which is thought to release acetylcholine, is unable to exert a strong and continued action on the formation of acetylcholine by tissue slices, unless glucose has also been added to the saline medium [Mann *et al.* 1939]; and (3) as the accelerating action of ether on the formation of acetylcholine is also apparently on the release of acetylcholine, the fact that glucose accelerates synthesis by fresh tissue in an ether-saline medium is also in favour of this assumption.

Against these points of favourable evidence must be placed the fact that glucose does not accelerate synthesis of acetylcholine by brain powder, either in saline or in ether-saline. Unlike respiring fresh brain, however, with its other large metabolic demands, the dried powder may need only minute amounts of glucose for the synthesis, and it may not be possible to reduce the glucose content in this system below the effective level, without similarly affecting other substances also necessary for the synthesis, but not replaceable by glucose.

The inhibiting effect of excess of glucose may well be on the release of acetylcholine, affecting the synthesis only indirectly. The absence of this effect in ether-saline could thus be explained. It is unlikely that glucose would be able to counteract efficiently the readier release brought about by the lipid solvent action of ether. Mann *et al.* [1939] have shown that the optimal concentration of glucose for synthesis of acetylcholine by fresh brain tissue is well below that of normal blood; at the concentration characteristic of normal blood, glucose is already inhibitory in action. If we may apply results obtained in saline suspensions to the conditions *in vivo*, we may suppose that the blood glucose would normally exert a similar restraining effect on the continuous release or synthesis of acetylcholine in the central nervous system. We know, from the central actions of artificially applied acetylcholine, and of eserine, that accumulation of acetylcholine in the motor cortex [Miller, Stavraky & Woonton, 1940] or in the spinal cord [Bülbring & Burn, 1941] causes a discharge from the motor neurons. The supposition is, therefore, not entirely unfounded, that when the glucose 'brake' is removed and the synthesis and release of acetylcholine allowed to proceed at an abnormal rate, increased motor activity may be expected. The convulsions associated with the lowering of the blood glucose by insulin might find an explanation on these lines. Previous attempts have failed to correlate these convulsions with acetylcholine. There was no change in the acetylcholine content of the brain [MacIntosh, 1939] or in the synthetic activity of the minced brain [Mann *et al.* 1939] from an animal which had died in insulin convulsions; and no changes of this kind would, in fact, be expected in the light of the above suggestions concerning the effects of glucose, at different concentrations, on the synthesis and the release of acetylcholine from the brain cells.

An inhibiting action of glucose on the release of acetylcholine was apparently observed, but disregarded, by Solandt and myself [1942] when analysing the stimulating action of glucose on an intestine preparation. Contrary to our expectations, perfusates obtained from the rabbit's intestine did not contain more but less acetylcholine when the perfusion fluid contained 0·1% glucose. The combined acetylcholine output in three perfusions was over 30% greater without glucose than in corresponding experiments with glucose in this concentration. The result was not followed up on account of the great individual variations. It is tempting also to correlate the 'hunger contractions', associated with a lowering of blood glucose, with a removal of the 'brake' on the release of acetylcholine in the stomach wall.

Ether. If we assume that the tissue acetylcholine is bound to some cell constituent, the lipids may be necessary for protecting the complex against the tissue cholinesterase. In dissolving the tissue lipids, the ether would remove this protection, and the acetylcholine held in the tissue would consequently diminish and finally disappear. This was the effect of ether on a suspension of brain tissue, ground and incubated with ether but without eserine.

The accelerating action of ether on the synthesis of acetylcholine has been explained by Mann *et al.* as being analogous to that of potassium. Their main evidence was based on the finding that ether diminished the acetylcholine held in the tissue. The experiments with brain powder have given a different result. When kept in eserine-saline without ether, the powder was unable to build up a store of tissue acetylcholine, but it acquired this property in the presence of ether. This observation, however, is not contradictory to the assumption that ether in the presence of eserine may also facilitate the release of acetylcholine, and that this may be even its most important action in the system containing fresh tissue. In the system made from the dried powder, however, where release from the particles in the absence of ether is already in excess of synthesis, so that no accumulation in the particles occurs, the direct promotion of synthesis by ether becomes clear; in its presence the particles also acquire a store of acetylcholine. Certain effects of ether, here described, must remain subjects for further experiment, rather than for discussion at the present stage.

SUMMARY

1. Some acetylcholine is released and replaced by synthesis in fresh brain during grinding. These processes continue in a suspension of the brain material in eserine-saline at 36° C., so that acetylcholine increases in the system while the quantity in the tissue particles remains practically constant.

2. Dried and powdered brain substance, suspended in saline, is still capable of synthesis of acetylcholine. If eserine is present, acetylcholine accumulates in this system also.

3. The effects of ether, on the synthesis of acetylcholine in saline suspensions of fresh and dried brain substance, are described.

4. In a system composed of dried brain, saline, eserine and ether, synthesis of acetylcholine at room temperature is greater than under any other conditions so far observed.

5. The effects of temperature, oxygen, glucose, cyanide, iodoacetic acid, K^+ and Ca^{++} on the synthesis of acetylcholine in some or all of these artificial systems containing brain tissue are described and discussed. The inhibitory effect of glucose at its normal concentration in the blood is discussed in relation to the effects of hypoglycaemia.

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EXCITABILITY CHANGES AT THE NEURO-MUSCULAR JUNCTION DURING TETANY

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A great part of the muscular activity during tetany is known to be of peripheral origin, but it is not yet clear how the components of the neuro-muscular system are affected in that condition. The main object of the present investigation was, therefore, to distinguish between the activity of the nerve endings, end-plates and muscle fibres during tetany.

Tetany has been induced either by removal of the parathyroid glands or by reduction of the ionized calcium in the tissues of cat and frog preparations with intact circulation. Symptoms produced by these two methods show a remarkable similarity. The tetany-like condition due to calcium lack can be quickly and conveniently obtained. Furthermore, the effect of calcium reduction on the neuro-muscular junction has already been studied in detail in isolated frog's muscle. There, the calcium content in the surrounding tissue can be estimated accurately, and the experiments on the single nerve-muscle fibre preparation yield information more easily about the state of the muscle membrane [Kuffler, 1944]. These findings, if correlated with the present experimental results in animals with intact circulation, give a fairly satisfactory explanation of the activity at the nerve-muscle junction during tetany.

Some evidence has also been obtained of activity originating at the central synapses, but these are not at present accessible to direct investigation, and, therefore, the scope of the paper has been limited to the peripheral component, the neuro-muscular junction.

METHODS

The experiments were performed on cats and frogs, and nerve-muscle preparations with intact circulation were used. The parathyroids, together with thyroids, were aseptically removed in eleven cats, and, in addition, as much as possible of the accessory parathyroid tissue in the neck region and that attached to the thymus was taken out. At the same time, the sciatic nerve was cut on one side in nine of the cats. For electric recording the cats were set up in a warm moist chamber kept at 37° C., as described by Eccles & O'Connor [1939]. Most of the observations were made on the hind legs.

Summer frogs (*Hyla aurea*) were used in over forty experiments. There are usually two parathyroids on each side about 0·5 mm. in diameter. They are not attached to the thyroid glands, and can be removed with the aid of a binocular dissecting microscope, as described by

Waggener [1930]. Their position varies greatly, and they are frequently found near to the ventral branchial body (ventraler Kiemenrest), or occasionally farther cranially in the vicinity of the vena jugularis externa. In many preparations they could not be located at all, mainly on account of accumulation of fatty tissue in the neck and precordial region. Slight bleeding during the first stages of the operation also makes the successful location of the parathyroids very difficult or impossible. In three cases histological examination showed that the tissue removed contained, the parathyroid glands. Sepsis was slight or absent, and, when tetany did not occur, the animals lived for many weeks afterwards. Light ether anaesthetic was found most convenient, and the frogs recovered after the operation in 10–30 min. On account of their small size, not more than 1·0 c.c. of blood could be obtained from the heart of the frogs at the end of the experiments, therefore only a few blood-calcium estimations could be made.

RESULTS

Tetany in cats

In the eleven cats from which the thyroids and parathyroids were removed very varied symptoms developed after 20–30 hr. Five cats (1, 2, 5, 7, 8, Table 1) showed twitchings all over the body, with periodic tonic and clonic contractions which became very pronounced when the animal tried to walk. The cats sometimes had difficulty in breathing, became unable to stand up, and showed a series of symptoms similar to those previously described for cats and other animals [Thomson & Collip, 1932; West, 1935; Greenberg, Boelter & Knopf, 1942]. Three of the cats showing severe symptoms died, presumably during an attack at night time, before electric recordings were done. Four cats (6, 9, 10, 11, Table 1) were less affected, showing occasional twitchings, tremblings and shivering after exertion, repeated shaking of the paws and of

TABLE 1. Symptoms in cats after removal of thyroids and parathyroids

	Spontaneous macroscopical symptoms on day of experiment or death		Symptoms during anaesthesia. Muscles exposed					
			Nembutal		Ether			
Cat no.	Days after operation	Severity of tetany	Fibrillation	Fasciculation	Fibrillation	Fasciculation	Decerebration	Serum-calcium mg./c.c.
1	3	+ + +	+ + +	+	–	–	—	4·7
2	(Died 2 days after operation)	+ + +	–	–	–	–	—	—
3	7	No symptoms	–	–	+ + +	–	—	—
4	7	No symptoms	+ + +	+	–	–	—	5·0
5	(Died 7 days after operation)	+ + +	–	–	–	–	—	—
6	1	+ +	–	–	+ + +	+	Fibrillation tonic-clonic contractions	5·0
7	(Died 7 days after operation)	+ + +	–	–	–	–	—	—
8	4	+ + +	–	–	+ + +	+ +	—	4·5
9	4	+ +	–	–	+ + +	+ +	Fibrillation tonic-clonic contractions	5·0
10	9	+	–	–	+ + +	+	Do.	4·5
11	9	+ +	+ + +	+	–	–	—	3·5

the head. Two cats (3 and 4) showed no signs of abnormality. In all the nine animals in which the sciatic nerve was cut during the operation, no macroscopically visible spontaneous activity developed in the muscles of the denervated limbs. Eight of these cats showed spontaneous activity in the other muscles whose nerves were intact.

During the final experimental recordings in two cats (1 and 11) which were anaesthetized with nembutal, it was found that the macroscopically visible signs of tetany disappeared. No spontaneous activity was observed through the skin. When, however, the muscles were exposed, close observation showed continuous fibrillation and intermittent small twitches in most muscle groups of the legs. The fibrillary discharges were also recorded electrically. They persisted undiminished after cutting the nerves leading to the muscles under observation. The activity was due to single muscle fibre discharges. This could be established from the small size of the impulses, from the asynchronous activity of the different units and also from comparison with denervated fibrillating muscles. In normal cats spontaneous fibrillation was never observed under nembutal anaesthesia.

The term 'fibrillation' is used to describe the activity of individual muscle fibres, while 'fasciculation' is used for synchronous discharges of small groups of muscles fibres (small twitches), presumably set up by nerve impulses [cf. Denny-Brown & Pennybacker, 1938].

The other animals were anaesthetized with ether which also abolished the spontaneous and macroscopically visible activity during tetany. When the skin was opened and the muscles inspected, fibrillation and occasional fasciculation were still observed. During the decerebration which followed in three cats, abnormal bleeding seemed to occur, attributable, presumably, to the calcium lack. This was successfully checked by application of thrombin to the brain stem. The coarse muscle activity returned 20–30 min. after decerebration. Large twitchings and sometimes tonic-clonic contractions could be seen, especially in the tibialis muscles (cats 6, 9, 10, Table 1). Such activity was usually enhanced by handling the muscles, or after indirect stimulation through the nerves. It did not persist continuously, but alternated with comparatively quiet periods during which only occasional twitches were observed on a background of almost continuous fibrillation. All three types of activity, twitchings, tonic-clonic contractions and fibrillation were sometimes seen simultaneously in different muscles of the same limb. In these preparations, as in the two Nembutal experiments, fibrillation persisted after severing of the nerves or blocking them with cocaine, and, as a rule, occasional twitchings were also seen. However, 4–6 days after denervation no twitchings, only fibrillation, could be detected in the exposed muscles.

It is, therefore, clear that fibrillation occurs during tetany, and this activity can persist without discharges from the nerve-endings reaching the endplates.

It also seems that fibrillation is the most readily produced symptom for, in the two parathyroidectomized animals which apparently showed no signs of tetany (cats 3 and 4), fibrillation was observed when the muscles were exposed seven days after the operation.

Blood serum calcium. In seven cats with symptoms of tetany the serum-calcium was determined. In six (Table 1) it was between 4·5 and 5·0 mg./100 c.c. and in one it was 3·5 mg./100 c.c. Normal controls showed 9–10 mg./100 c.c. Cat 4 had only the slightest signs of tetany, and cat 11, with the lowest serum-calcium, did not develop severe symptoms. This confirms numerous other findings that a low blood-calcium value does not necessarily result in a condition of severe and active tetany. It is probably the lowering of the ionized fraction of the calcium in the blood which is important in causing the nervous symptoms in tetany (cf. later).

Tetany in frogs

Tetany in frogs was induced by (i) removal of the parathyroids, (ii) injection of small quantities of sodium citrate. Waggener [1930] showed that tetany occurs in frogs after parathyroidectomy, and that the animals die in approximately 3 days. Such severe tetany was not observed in the present experiments, presumably on account of incomplete removal of all the parathyroid tissue. Only one frog died after severe symptoms of tetany. The other frogs did not develop spontaneous nervous activity visible to the naked eye, such as twitchings or clonic contractions. Most of them, however, showed a very great excitability when approached and seemed to be 'nervous' as compared with the normal control animals.

It was at first thought that the muscles of the frogs could be isolated and placed immediately afterwards into liquid paraffin in which electric recording could be done without undue drying of the muscles. Soaking in the usual normal saline solution would thus be avoided. This, however, was not practicable, because even normal muscles, if placed into paraffin immediately after dissection, develop neuro-muscular block, and then become inexcitable even to direct stimulation. This is probably the phenomenon shown by Duliere & Horton [1929] to be due to potassium leakage from the muscle fibres. The excitability of such isolated muscles from normal or tetany-frogs is restored after bathing the preparation in Ringer's solution for several minutes.

All electric recordings have therefore been made from muscles with intact circulation in frogs under ether anaesthesia. The animal was pinned down on a cork-board, the sartorius exposed and 3–4 mm. of its nerve freed for indirect stimulation. Recording electrodes could then be placed on any point of the muscle. To avoid drying, the skin was pulled over the exposed muscles between observations.

Fig. 1*a* shows a typical response of such a preparation after indirect stimulation through the nerve. The large spike action potential (40–50 mV.), as usually recorded from the sartorius, is followed by a great number of smaller responses. In a fresh preparation which has not been frequently stimulated such discharges may continue for several seconds. Continued stimulation eventually abolishes the repetitive discharges, and a partial neuro-muscular block develops. The long lasting discharges (Fig. 1*b*) are shown to be composed of single muscle fibre responses by their size, by the regular frequency of the units and especially by comparison with impulses which are set up by acetylcholine application, when the nerve endings do not discharge [Kuffler, 1943]. When both recording electrodes are placed in close proximity to the pelvic, nerve-free, end of the sartorius it can be observed, from the polarity of the action potentials, that the impulses originate in the innervated portion of the sartorius. Stretching or touching the muscle also sets up frequent bursts of single fibre responses, starting at the innervated muscle regions.

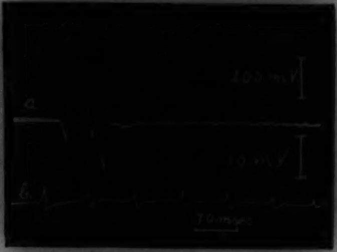

Fig. 1. Action potentials recorded from a circulated preparation of frog's sartorius under ether anaesthesia. (*a*) Response after nerve stimulation shows the large muscle action potential followed by smaller discharges. (*b*) Several seconds later single muscle fibre responses are still observed. Note the different amplification in *a* and *b* (see text).

Thus, it seems clear that the endplate regions in such parathyroidectomized frogs are abnormally sensitive to the stimulating action of nerve impulses, and also to mechanical stimulation such as stretch and touch.

In most frogs which had their parathyroids removed, but showed no visible symptoms, tetany could be easily precipitated by doses of sodium citrate which were too small to have a detectable effect on normal animals (cf. later). An injection of 0·2–0·4 c.c. of 2·5% sodium citrate was made into the dorsal lymph-sac of the animals. Tetany of varying intensity developed in 5–10 min., and, for several hours after the injection, sporadic twitchings could be observed, especially in the extremities. Some frogs returned to normal afterwards, but others showed intermittent attacks for as long as 18–20 days. These attacks developed sometimes without apparent provocation, and consisted in twitchings of the toes or of bigger muscle groups. Tonic contractions were also frequently observed, and the frogs had evident difficulty in breathing. But usually the violent attacks of tetany could be produced by simply touching or picking up an animal, especially after it had been at rest for some hours. When the symptoms of tetany disappeared altogether, they could be easily renewed by injection of additional sodium citrate. Ether always abolished these macroscopically visible nervous symptoms, but they usually returned immediately as the frog recovered from the anaesthetic.

In addition to removal of the parathyroids, one of the sciatic nerves was cut or crushed in fifteen frogs, and the development of tetany in such a denervated limb was compared with the normal one. Two of these frogs were kept for 18 and 20 days and showed signs of tetany as described above. In addition to observation through the skin, the muscles were frequently exposed and observed under a binocular dissecting microscope, and the skin was closed again without causing sepsis or other great damage. This was repeated as frequently as 10 times, and such close observations of the exposed *denervated* limb muscles could be performed without anaesthetizing the frog. All the activity in muscles with their central connexion severed must have been of peripheral origin, arising at nerve endings, endplates or the rest of the muscle fibres. The latter seems unlikely as all the repetitive discharges after nerve stimulation, or stretching and touching came from the innervated portion of the sartorius (cf. above). It is at times very difficult to distinguish by observation through the microscope between activity set up by discharge of nerve fibres or of single muscle fibres. However, after frequent observation of fibrillary activity in denervated muscles or of the single fibre discharges set up by drug application [Kuffler, 1943] and also after immersion in calcium-free Ringer's solution [Kuffler, 1944], fibrillation could be distinguished from fasciculation with reasonable certainty. For approximately 24 hr. after nerve section, spontaneous fasciculation (sudden contractions of small groups of muscle fibres) could be observed in frogs with general symptoms of tetany. The activity, however, was much weaker, and the discharges were less frequent than in the opposite leg with intact innervation. Together with the intermittent fasciculation, a more continuous and persistent fibrillation could be seen in most denervated leg muscles. After 24 hr. no spontaneous activity was observed (the frogs were of course not watched continuously), but fibrillation, lasting for several seconds, could easily be produced in such muscles by active movement or by electric stimulation. In two preparations, in which indirect excitability through the nerve ceased 8 and 12 days after denervation, strong fibrillation lasting for 2–3 min. could still be induced by electric stimulation or by touching the muscle with electrodes or a small rod. The muscles of the other extremity, which had not been denervated, responded to nerve stimulation with repetitive responses as seen in Fig. 1.

It thus appears that, in a muscle which does not receive impulses from the central nervous system, two types of spontaneous response can be set up under conditions of tetany, (i) fibrillary activity, (ii) fascicular activity due to discharges of single nerve fibres or groups of fibres. This latter activity ceases long before the nerve has fully degenerated. It is also much less persistent, and is never observed without fibrillation going on at the same time.

Tetany can also be induced by injection of sodium citrate alone into normal, unoperated frogs. In ten frogs such tetany was produced. The best method was injection of small quantities (0·2–0·4 c.c. of 2·5% sodium citrate twice daily for several days) into the dorsal lymph-sac. Larger quantities frequently paralysed and killed the animals within 10 min. Once tetany developed, it could not be differentiated macroscopically or electrically from the condition after removal of the parathyroids. The frogs, however, recovered completely from this type of tetany within 1–2 days. The difference between the latter and parathyroidectomized frogs therefore was (i) a much greater amount of sodium citrate was required to induce active spontaneous tetany, (ii) the symptoms of tetany disappeared more quickly (1–2 days as compared with as long as 20 days).

Discussion

Experimental tetany has been produced frequently in many types of warm blooded animals [for an extensive review see Thomson & Collip, 1932]. It is generally agreed that the symptoms are to a great extent of peripheral origin, and some investigators also found evidence for a central component [Paton, Findlay & Watson, 1917; Greenberg *et al.* 1942].

The present experiments demonstrate the important rôle played by the endplate region during two types of tetany, after removal of the parathyroid glands, which is followed by a diminution of serum-calcium and after reduction of the ionized calcium in the blood-stream by sodium citrate. In frogs, the course of tetany affecting the peripheral component alone can best be observed in muscles deprived of their central connexions. Under such conditions, spontaneous activity arose at the innervated part of the muscle. At times it was entirely due to single muscle fibre discharges from the endplate region without accompanying nerve impulses, but, generally, larger groups of muscle fibres also showed activity, presumably fired off by nerve impulses. The endplate seems to be in a state of raised excitability even when no spontaneous activity can be observed. A nerve volley sets up discharges for several seconds from the endplate regions. The potentials immediately (for 50–70 msec.) following the main spike (Fig. 1*a*) are due to groups of muscle fibres, probably discharged by repetitive nerve impulses. The small potentials recorded for some seconds after a nerve volley (Fig. 1*b*) cannot, however, be set up by nerve impulses, since they are single muscle fibre responses. The discharges are not due to a greater production or persistence of the "transmitter" after nerve stimulation, as they arise also after stretching of the muscle, or after direct stimulation of the pelvic, nerve-free, end of the sartorius, when no nerve impulses are set up. Moreover, slight touch or pressure with a glass rod, which is normally ineffective, sets up such a train of impulses. This is in good agreement with findings on the isolated sartorius and in single nerve-muscle fibre preparations in which the effect of calcium reduction could be studied in detail [Kuffler, 1944]. There, calcium lack affected the endplates before the other structures, and direct evidence showed that spontaneous activity never resulted at the nerve-free parts of muscle fibres.

Thus, by injection of sodium citrate into the circulation, or by reducing the calcium in the fluid bath in isolated muscles, a set of symptoms can be produced which, with the present methods of investigation, seem indistinguishable from those appearing after total or partial removal of the parathyroid glands.

In cats, the coarse activity of muscles is accompanied by nerve discharges, since, during tetany, impulses can be recorded from the ventral roots [Harvey, 1944]. The similarity of the general findings in cats and frogs is very striking, and it is suggestive that the spontaneous fibrillation in both, not in frogs only, originates at the endplates and not anywhere along the muscle fibres.

A general lowering of the threshold of excitation at all or numerous junctional regions, including central synapses, could be expected to produce a picture similar to that of tetany. Not only would the peripheral junctions receive more discharges from the central regions, but they would respond repetitively to each impulse. It would seem that in tetany the spontaneous discharges start mainly at the synapses and, secondarily, at nerve endings. This is the case at the neuro-muscular junction, where fibrillation may persist continuously, while fasciculation appears only intermittently in muscles with their central connexions cut.

SUMMARY

1. Experimental tetany has been produced in cats, by removal of the thyroid and parathyroid glands, and in frogs, by removal of the parathyroids only and also by reduction of the ionized calcium in the bloodstream. The effect of tetany on the neuro-muscular system has been studied in detail.

2. The symptoms of tetany show, in both types of animals, a great range of severity related, presumably, to the completeness of removal of the parathyroid tissue.

3. In muscles with their nerves cut, two types of spontaneous activity can be observed: (*a*) fibrillation, due to single muscle fibre discharges originating at the endplate regions; (*b*) synchronous contractions due to simultaneous discharges of groups of muscle fibres, presumably set up by discharges at the nerve endings.

4. The endplate region is in a state of hyperexcitability, even when spontaneous activity of the muscles is absent. A nerve volley arriving at the

nerve-muscle junction sets up at this point discharges lasting up to 3–4 sec. This long lasting part of the activity is not accompanied by nerve impulses. Stretch of the muscles, or slight pressure, initiates a similar activity.

5. The blood-calcium content of seven operated cats was reduced to about half normal. The severity of symptoms was not directly related to the degree of blood-calcium reduction.

6. It is suggested that the symptoms of tetany are a result of lowering of the threshold at synapses and endplates.

I wish to thank Dr A. M. Harvey for much advice and also Mr R. N. Lyons for his help in determining the serum-calcium in the animals.

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EFFECT OF THE INITIAL LEVEL OF THE BLOOD PRESSURE UPON THE RESPONSE OF THE HUMAN SUBJECT TO BLOOD PRESSURE RAISING REFLEXES

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(*Received* 23 *May* 1944)

In studies concerning the functional pathology of arterial hypertension, observations are often quoted in which the effects of procedures which raise or lower the blood pressure are compared in hypertensive patients and healthy controls. Where the magnitude of the induced change in blood pressure differs in the two groups, there remains usually some doubt whether the difference is due to a fundamental alteration of the reaction of the blood vessels in the hypertensive patients or, alternatively, is merely the result of a higher initial level of the blood pressure. Thus, we find the suggestion made that, in essential hypertension, the increased response to the cold pressor test of Hines & Brown [1932] is the result of the high initial level of the blood pressure and not of any inherent change in the reactions of the blood vessels or of the nervous system which has been brought about by a pathological process. It is of some interest in this connexion that the pressor effects of drugs depend to some extent upon the initial state of the blood vessels. Thus, in animal experiments, rises of blood pressure caused by adrenaline are commonly less when the initial blood pressure is high.

It was decided, therefore, to study in normal subjects the effects of changes induced in the level of the blood pressure upon the magnitude of the response to a pressor reflex.

METHODS

Blood pressures were measured with a mercury sphygmomanometer and by the methods recommended in the joint report of the committees appointed by the Cardiac Society of Great Britain and Ireland and the American Heart Association [1939]. Two pressor reflexes were employed: the cold pressor test of Hines & Brown [1932] and the pressor reflex from muscle described by

Alam & Smirk [1937]. The cold pressor test is performed by arresting the circulation through an arm by means of an inflated sphygmomanometer cuff round the upper arm. During the period of circulatory arrest the hand and wrist of the ischaemic arm are plunged into water at 4° C. This leads reflexly to an increase of the general blood pressure which may be measured in the opposite arm, where the circulation is free. When repeated under standard conditions such increases of the blood pressure are usually of similar magnitude.

The pressor reflex from muscle is elicited by arresting the circulation through an arm or leg by means of an inflated sphygmomanometer cuff round the upper arm or thigh. Exercise of the ischaemic forearm or calf muscles is then carried out, by opening and closing the hands or by raising and lowering a weight which rests on the knee, the subject being seated. The metabolites formed during this exercise accumulate in the ischaemic muscles and lead, reflexly, to an increase in the general blood pressure which persists so long as the circulation in the limb remains arrested. When the circulation is restored, so that the accumulated metabolites are dispersed, the blood pressure falls back to normal. Repetitions of the reflex, with a standard exercise of the limb, produce similar increases of the blood pressure.

The response of each subject to the cold pressor test was determined, first with the initial blood pressure at its usual level and secondly after the blood pressure had been elevated by administration of adrenaline or ephedrine. Adrenaline was given subcutaneously in doses of from 0·3 to 1·9 mg., the dose being judged by a preliminary experiment and in each case being sufficient to produce a definite rise in the blood pressure. The ephedrine, in all cases, was used in a dose of 33 mg. Another method was to raise the initial blood pressure by one of the above mentioned pressor reflexes, and then, while the pressure was thus maintained reflexly at a high level, to determine the response to a further application of the cold pressor test. In a few experiments the magnitude of the response to the pressor reflex from one group of muscles (say the forearm muscles) was tested before and after the blood pressure was elevated by eliciting the reflex from another group of muscles (say the calf muscles).

RESULTS

Effect of a rise of blood pressure induced by adrenaline or ephedrine upon the response to the cold pressor test

It will be observed in Figs. 1 and 2 that increases in the systolic and diastolic pressures produced by the cold pressor test are less in the experiments where the blood pressure has already been raised by adrenaline or ephedrine than in the experiments where the cold pressor test was applied with the blood pressure at its normal level.

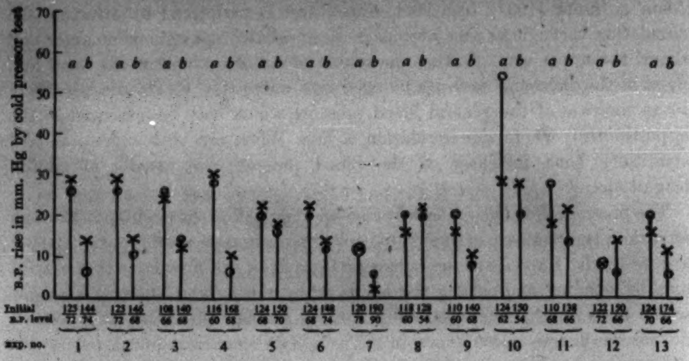

Fig. 1. The effect of the cold pressor test on the blood pressure of the normal human subject: (*a*) when the initial blood pressure is at a normal resting level; (*b*) when the initial blood pressure is raised by adrenaline subcutaneously. Crosses indicate rises of systolic pressure. Circles indicate rises of diastolic pressure.

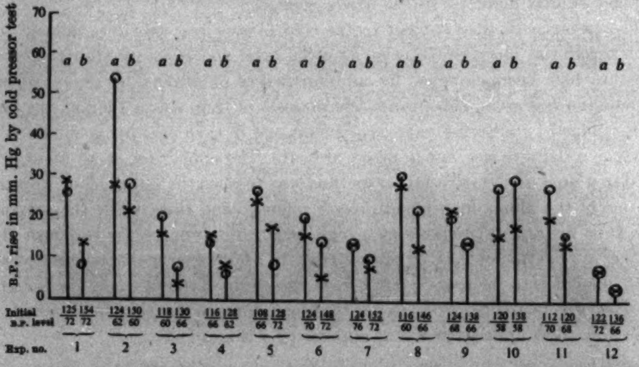

Fig. 2. The effect of the cold pressor test on the blood pressure of the normal human subject: (*a*) when the initial blood pressure is at a normal level; (*b*) when the initial blood pressure is raised by ephedrine subcutaneously. Crosses indicate rises of systolic pressure. Circles indicate rises of diastolic pressure.

Effect of a rise of blood pressure induced reflexly upon the response to the cold pressor test

It will be observed from Fig. 3 that in nine out of ten instances the pressor response, obtained when the initial blood pressure had previously been raised by the pressor reflex from muscle, was appreciably less than in the experiments where the blood pressure started from a normal level. The rise in blood pressure in response to the muscle reflex elicited from the forearm muscles was also less if the blood pressure was first elevated by exercise of the ischaemic calf muscles. Evidently the effect on the blood pressure of two pressor reflexes evoked together on the same individual is less than the sum of the effects of the two reflexes acting separately.

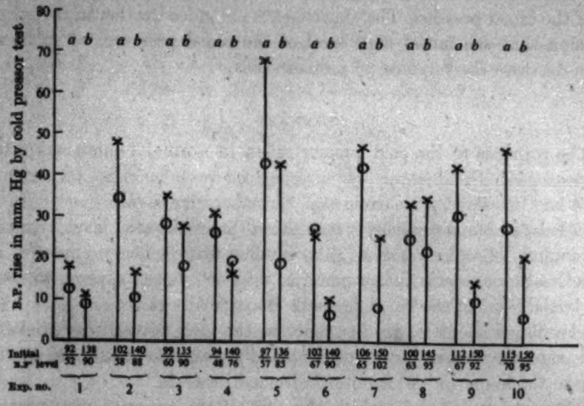

Fig. 3. The effect of the cold pressor test on the blood pressure of the normal human subject: (*a*) when the blood pressure is at a normal resting level; (*b*) when thei nitial blood pressureis raised by a reflex from ischaemic muscle. Crosses indicate rises of systolic blood pressure. Circles indicate rises of diastolic blood pressure.

Discussion

It has been shown in a series of normal subjects that the response to the cold pressor test of Hines & Brown (elicited from an arm) is lessened in those experiments where the blood pressure is first raised by adrenaline or by ephedrine or by means of the pressor reflex from muscle. Such results are in accord with pharmacological experiments which show that substances raising the blood pressure usually act more powerfully when the blood pressure is low than when it is high. These observations would lead one to expect, in general, that the response to pressor stimuli should be *less* when the blood pressure is high than when it is low.

It has been shown by Hines & Brown, however, that the rises of blood pressure from the cold pressor test are, in general, greater in essential hypertension than in health. Similar observations were made by Alam & Smirk using the cold pressor test and also the pressor reflex from voluntary muscle.

The responses to both types of pressor reflex, however, were no greater in cases of renal hypertension than in normal individuals and probably were less.

It is evident that in certain instances, where the level of blood pressure has been raised experimentally, the response to the cold pressor test is reduced during the period of blood pressure elevation. It does not appear justifiable, therefore, to attribute the large blood pressure rises, obtained by the cold pressor test in cases of essential hypertension, to the effect of the high initial level of the blood pressure. The experiments are indeed rather in favour of the conclusion that the initial high level of the blood pressure, as such, would tend to decrease the response to pressor reflexes.

Summary

1. The response to the cold pressor reflex in normal human subjects has been measured:

(*a*) When the blood pressure is at a normal resting level.

(*b*) When the blood pressure is maintained at an elevated level by means of (1) adrenaline, (2) ephedrine, or (3) by a reflex from ischaemic muscle.

2. In healthy subjects the response to pressor reflexes appears to decrease as the initial level of the blood pressure is artificially raised.

3. The observations lend no support to the idea that, in essential hypertension, the enhanced reactivity of the blood pressure to pressor reflexes is due merely to the higher initial level of the blood pressure.

4. Instances are cited in which the sum of the separate responses of an individual to two blood pressure raising stimuli, applied at different times, was found to be greater than the response to the same two stimuli, applied simultaneously.

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RESPIRATION AND CARDIAC OUTPUT IN THE ISOLATED HEART-LUNG-HEAD PREPARATION IN THE RAT

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(*Received* 24 *June* 1944)

Two of the major circulatory problems in the mammalian organism, at rest and during muscular exercise, are first the mechanism whereby the blood is returned from the periphery to the right side of the heart and secondly the means whereby peripheral resistance maintains a sufficient arterial blood pressure to prevent anoxia of heart muscle. In view of the normally rapid rate of the heart of the rat, about 400 beats/min., it was thought worth while to determine whether, in such a rapidly beating organ, the factors determining auricular inflow and ventricular output are the same as those obtaining in the heart of the dog, and further to determine the part which changes in both positive and negative ventilation play in affecting cardiac inflow and output.

METHODS

The isolated heart-lung and heart-lung-head preparation

The isolated heart-lung preparation in the rat has been in use in this department for several years. It proved to be of considerable utility in an investigation of fat utilization in the aglycaemic rat [Cruickshank & Kosterlitz, 1941], and, since the details of the preparation have never been published, a brief note on the method may be of value.

Lister strain rats of between 300 and 400 g. weight were used and were fed on an ample diet. The hearts of experimental animals weighed from 0·7 to 1·2 g. and made excellent preparations which, with an arterial blood pressure of 80–100 mm. Hg, a venous pressure of 70 mm. of blood, a respiration rate of 72 per min., a tidal air volume of 2–4 c.c. and a blood temperature of 38° C., would continue to function almost unchanged for four or more hours. Amytal 0·01 g./100 g. rat was used as the anaesthetic.

Blood for the preparation is obtained from rats, the number depending on the amount of blood required. Bleeders weighing about 350 g. will give 8–10 c.c. of blood from the carotid. To do this the rat is secured to the animal board, the right carotid carefully exposed and cut between ligatures. The artery having been cleared of tissue, the rat is placed vertically, head down; the vessel, steadied by forceps holding the ligature, is opened by partially cutting through it with fine scissors and the blood is collected in a 30 c.c. beaker to which 100 Toronto units Heparin B.D.H. per 10 c.c. blood have been added.

In making an isolated heart-lung preparation the chest is opened carefully in the mid-line, the thymus retracted upwards and the arch of the aorta exposed. In earlier experiments the azygos vein was clamped at its junction with the left superior vena cava. This was later found to be unnecessary. The right superior vena cava is ligated and a loose ligature is placed round the inferior vena cava above the diaphragm. The inferior vena cava is ligated below the diaphragm and the cannula inserted also from below the diaphragm. The azygos vein and oesophageal veins are now tied. The innominate artery is ligated, and a ligature, placed round the aorta, is pulled upon to prevent bleeding while a cannula is inserted into the aorta. As soon as the circulation is established the resistance pressure is raised to bring the arterial blood pressure up to 80 mm. Hg and the venous pressure is set at about 70 mm. of blood. From the diagram in Fig. 1 the general set up will be clear. The bore of the tubing is 4 mm., the venous reservoir can be of 5–50 c.c. capacity depending upon the type of experiment to be performed, e.g. where cardiac outputs are to be measured the reservoir should hold at least 30 c.c. of blood, where blood glucose is to be exhausted the total blood in circulation need not be more than 10 c.c. in which case the venous reservoir must be very small; a glass tube 5 in. long and ½ in. in diameter is ample and affords an accurate check on blood loss.

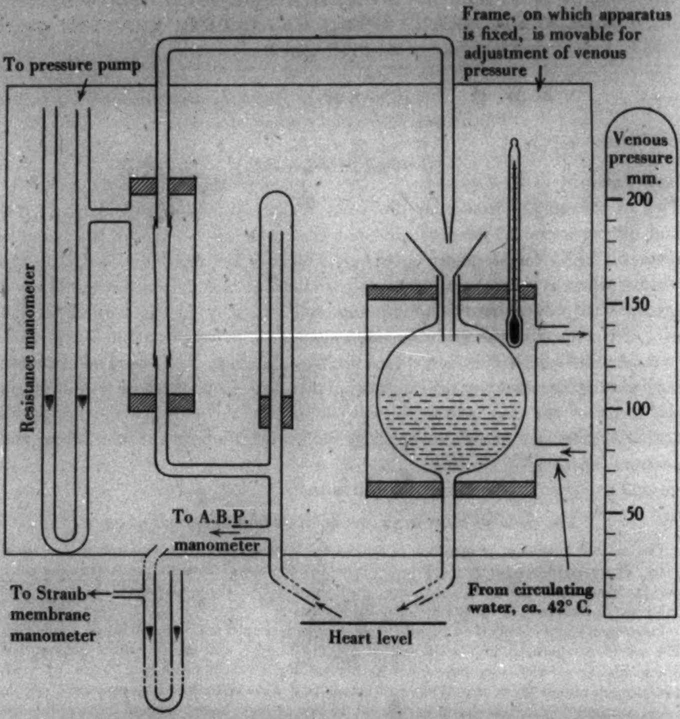
Frame, on which apparatus is fixed, is movable for adjustment of venous pressure
To pressure pump
Venous pressure mm.
200
150
100
50
Resistance manometer
To A.B.P. manometer
To Straub membrane manometer
From circulating water, ca. 42° C.
Heart level

Fig. 1. Diagram of apparatus for perfusion of isolated heart-lung-head preparation.

In making the isolated heart-lung-head preparation the arterial cannula is inserted first, a small part of the abdominal aorta just above the superior mesenteric artery being well cleared for its reception. The venous cannula should be inserted into the inferior vena cava just beneath the diaphragm, and ligatures tied both above and below the diaphragm; otherwise, blood will be lost by way of the phrenic arteries and their venous return into the inferior vena cava. When negative ventilation is to be used the diaphragm should be damaged as little as possible, and the ligatures about the diaphragm must be well placed or blood will leak into the liver and potential bleeding points will be opened up if or when negative ventilation is increased to give more than moderate inflation of the lungs. It is also important to ligate just above the diaphragm all veins passing through it into the abdomen. No bleeding points should be apparent before proceeding with negative ventilation.

The rate of the heart can be accurately recorded by the electrocardiograph or by photographing the moving lever of a Straub membrane manometer over the slit of any suitable photographic apparatus, such as the slit of the photographic box of an electrocardiographic machine. The membrane manometer is attached to the arterial blood-pressure manometer (Fig. 1).

Respiration

Positive ventilation of the lungs was carried out by means of a small Ideal Pump which was made for the purpose by Messrs Palmer and Co. Negative ventilation was performed by placing the animal in a small air-tight wooden box with holes appropriately placed for conveying arterial blood out to and venous blood in from the circulatory schema shown in Fig. 1. This is a modification of the design described by Daly, Elsden, Hebb, Ludany & Petrovskaia [1942]. The lid of the box consists of plate glass sealed into a wooden frame which is tightly screwed down on to a rubber gasket by wing nuts. A reduced pressure was created in the box by interrupting, at predetermined frequencies, the suction created upon the box by an Edwards' filter pump. Any effective suction apparatus such as a Cenco vacuum pump may be used. The method adopted for breaking the stream of air being sucked through the box was simple and capable of producing any degree

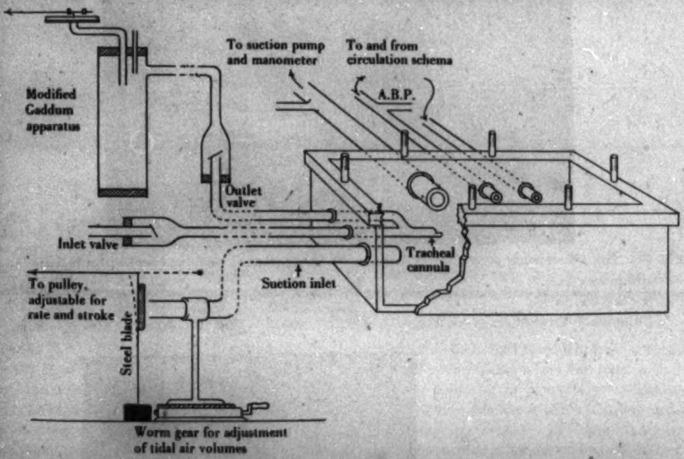
To suction pump and manometer
To and from circulation schema
A.B.P.
Modified Gaddum apparatus
Outlet valve
Inlet valve
Tracheal cannula
Suction inlet
To pulley, adjustable for rate and stroke
Steel blade
Worm gear for adjustment of tidal air volumes

Fig. 2. Diagram of apparatus for negative ventilation of isolated heart-lung-head preparation.

Fig. 3a. Typical tracing of the calibration curves of the modified Gaddum apparatus. Each step represents 1 c.c. tidal air at the respiration rate given at the top of the record.

Fig. 3b. Record showing constancy of pulmonary ventilation at varying respiration rates:

Frequency/min.	48	60	72	90	120	180
Stroke vol. in c.c.	6·3	5·0	4·2	3·3	2·5	1·7
Pulmonary ventilation in c.c./min.	302	300	302	300	300	306

Tidal Air c.c.	Pulmonary Ventilation c.c.
0·5	36
1·0	72
2·5	180
3·3	238
4·7	346

Fig. 3c. Typical record of negative ventilation. Respiration rate 72/min.

change in respiration rate, tidal air and pulmonary ventilation. These changes were effected by means of an ordinary 1 ft. steel ruler fixed at one end and bearing on the other a soft rubber pad which was allowed to close the air inflow tube at a definite frequency and for a definite time. By an appropriate pulley the steel ruler was caused to make suitable, limited excursions backwards and forwards, and the time of occlusion of the air inlet was arranged by having the air inlet tube firmly fixed to a fine worm screw by which it could be made to approach or be withdrawn from the rubber pad fixed on the end of the moving steel ruler. This simple device, shown diagrammatically in Fig. 2, proved most effective in determining the degree of inflation of the lungs.

Calibration of respiration records

The record of pulmonary ventilation was obtained by means of the Gaddum [1941] method as modified by Kosterlitz in this department. The valves were placed on the expiratory side and no trouble was occasioned by moisture. The record was made on a smoked kymograph drum. From the records of different degrees of pulmonary ventilation obtained at various frequencies (Fig. 3*a*) a calibration curve was drawn relating the change in mm. of the respiration record to the volume (c.c.) of the pulmonary ventilation. In the experiments to be described respiration rates varied from 48 to 150 per min., but it was clearly seen that rate did not affect the relationship between deflexion in mm. and pulmonary ventilation (Fig. 3*b*). Knowing the pulmonary ventilation and the rate of respiration, one could estimate the tidal air volume and check this against records taken when the Ideal pump was set for various tidal air volumes (see Fig. 4). In all experiments, the pump, with the inlet and outlet valves of the Gaddum apparatus in position, was calibrated in this way, and it is from a series of such calibrations (Fig. 3*a*) that the curve and graphs in Fig. 4 were constructed. Knowing the rate of respiration as set by the adjustable pulley one could therefore, as the experiment was being made, measure the tidal volume or, approximately, set the suction inlet tube to give any tidal volume required. Since tidal volume is a measure of the degree of inflation of the lung and therefore of the 'negative pressure' such a graph, relating tidal air volumes to rate of respiration, was of immediate value. An example of the type of record of negative ventilation obtained is given in Fig. 3*c*.

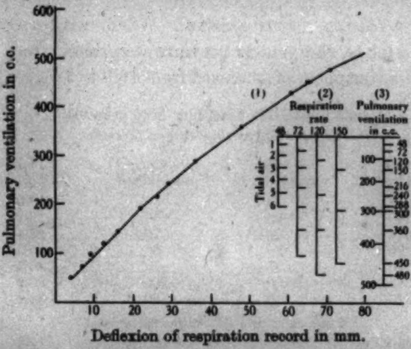

Fig. 4. Calibration curve of respiratory records. (1) Relationship between deflexion in mm. and pulmonary ventilation. (2) Graph for measuring tidal air. (3) Graph for measuring pulmonary ventilation.

RESULTS AND DISCUSSION

Circulatory factors affecting cardiac output

In all the numerous experiments made on the isolated heart-lung-head preparation the results have been very uniform and, accordingly, the data from one experiment are given to illustrate various points. Data from the innervated heart are immediately followed by those obtained when both vagus nerves, shown by electrical stimulation to be active, have been cut and the carotids ligated. Thus the results from the isolated heart-lung-head preparation are directly contrasted with those from the denervated heart or the isolated heart-lung preparation. Brief reference is made to the results because they corroborate on the rapidly beating heart what is known for the slower mammalian heart, e.g. the dog [Markwalder & Starling, 1914; Patterson & Starling, 1914].

The effects of increasing venous pressure. When, with a constant positive ventilation of the lungs, the venous pressure was raised from 50 to 150 mm. of blood, the cardiac output was increased from 16·0 to 38·4 c.c./min. The heart

TABLE 1. Isolated heart-lung-head preparation
Effect of increasing venous pressure

Rate beats/min.	B.P. mm. Hg	V.P. mm. blood	Output c.c./min.
216	73	50	16·0
240	75	60	19·2
264	80	70	23·4
270	85	80	25·2
276	85	90	26·4
288	90	100	30·0
312	120	150	38·4
Both right and left vagus nerves cut			
360	75	60	19·2
360	80	70	25·2
360	85	100	30·0
With arterial blood pressure constant			
228	85	40	16·8
264	85	70	22·8
276	85	80	26·6
300	85	100	32·4
Vagi cut			
360	90	40	14·4
360	90	50	19·2
360	90	70	24·0
360	90	100	33·6
Effect of changes in venous pressure in an isolated heart-lung preparation			
270	70	30	14·0
270	80	50	22·8
270	90	70	31·2
270	95	80	36·0
270	105	100	43·2
270	110	125	50·4
270	130	150	60·0
270	140	180	62·4
240	145	200	60·0

rate rose from 216 to 312 beats/min. accompanied by a definite rise in the arterial blood pressure from 73 to 120 mm. Hg. After the vagi were cut, the heart rate increased to 360 beats/min. and, with a venous pressure of 60 mm. blood, the output was 19·2 c.c./min., at 70 mm. 25·2 c.c./min., and at 100 mm. 30 c.c./min. These observations corroborate the findings of Bainbridge [1915], namely, that the venous pressure determines the rate of the heart by vagal pathways and is responsible for changes in cardiac output [see also Sassa & Miyazaki, 1920; and Anrep & Segall, 1926]. That these changes are due to venous pressure and not to changes in arterial blood pressure is also indicated in Table 1.

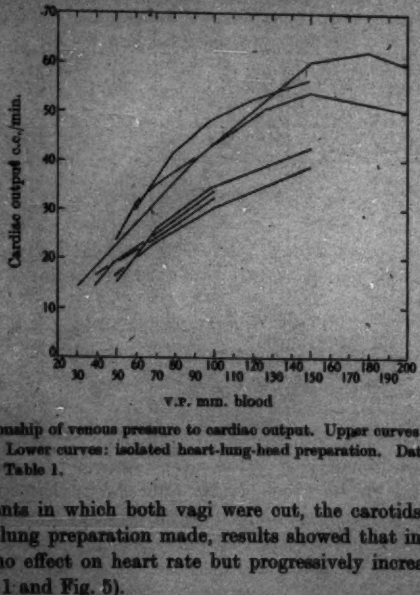

Fig. 5. The relationship of venous pressure to cardiac output. Upper curves: isolated heart-lung preparation. Lower curves: isolated heart-lung-head preparation. Data from one of each series only in Table 1.

In experiments in which both vagi were cut, the carotids ligated and an isolated heart-lung preparation made, results showed that increasing venous pressure had no effect on heart rate but progressively increased the cardiac output (Table 1 and Fig. 5).

Effect of increasing arterial blood pressure. The results of increasing arterial pressure, with venous pressure at 40 and 70 mm. blood, are shown in Table 2. Increasing arterial pressure from 50 to 140 mm. Hg has little, if any, effect on the cardiac output. On the other hand, the increase in arterial pressure occasions a very marked decrease in heart rate: with a low venous pressure, 40 mm., an increase in arterial blood pressure from 30 to 120 mm. Hg produces a 30% decrease in heart rate, viz. 360 to 252 beats/min. With a venous pressure of 70 mm. blood there is no material alteration in cardiac output until the arterial blood pressure passes 140 mm. Hg. There is again a marked change in heart rate, 348 to 246, which is abolished on cutting both vagus nerves. As venous pressures are increased to 100 mm. of blood a definite though not such a marked change in heart rate is seen and cardiac output does not change until blood pressures are above 140 mm. Hg. These results confirm the observations made on larger animals by Heymans [1925, 1929] and Anrep & Segall [1926]. It will be noted later that, with arterial pressures below 45 mm. Hg, the heart, despite optimal venous pressure, steadily deteriorates, doubtless owing to a poor coronary circulation.

TABLE 2. Isolated heart-lung-head preparation
Effect of raising arterial blood pressure at 40 and 70 mm. venous pressure

Rate beats/min.	B.P. mm. Hg	V.P. mm. blood	Output c.c./min.
360	30	40	27·6
324	60	40	27·6
276	100	40	27·6
252	120	40	27·6
348	50	70	36·0
342	65	70	36·0
312	95	70	36·0
288	110	70	36·0
282	115	70	34·8
276	125	70	36·0
258	140	70	34·6
246	145	70	32·4
246	155	70	26·4
Both right and left vagus nerves cut			
402	100	70	38·4
402	50	70	36·0
402	80	70	37·2
402	115	70	37·2
402	135	70	37·2
402	145	70	36·0
402	150	70	34·8

Effect of altering the heart rate. It has been shown that alteration of the heart rate within physiological limits does not *per se* alter the cardiac output. As long as there is ample diastolic time for venous filling, the venous pressure being constant, there is no change in cardiac output. The denervated heart, the rate of which has been increased, within wide limits, by adding adrenaline to the perfusing blood or decreased by cooling the auricle with ice, suffers no change in output (Table 3).

From the above results it may be concluded that heart rate does not affect heart output when venous pressure is between 40 and 70 mm. blood. Venous pressures below 30 mm. blood generally cause rapid failure of the heart from inadequate filling. At the other extreme, venous pressures over 150 mm. blood are not well taken, for the heart slowly dilates, and at a pressure of 200 mm. blood it quickly succumbs.

TABLE 3. Isolated heart-lung-head preparation
Effect of changes in heart rate on output. Both right and left vagus nerves cut

Rate beats/min.	B.P. mm. Hg	V.P. mm. blood	Output c.c./min.
Auricle cooled with ice			
420	100	70	38·4
408	95	70	38·4
318	95	70	37·2
300	90	70	36·0
282	90	70	34·8
270	90	70	34·8
Cooling stopped			
282	100	100	42·0
300	100	100	42·0
360	105	100	43·2
Heart rate increased by adrenaline			
312	110	80	28·8
336	110	80	28·8
390	115	80	30·0
Arterial blood pressure lowered			
390	65	80	28·8
390	50	80	28·8

Effect of occlusion of the carotid arteries. Just as the heart reacts immediately to an increase in arterial pressure so it responds quickly to a decrease in arterial pressure in the carotids caused by occlusion of these vessels. The data in Table 4 indicate this response and its absence when the vagi are cut. It should, however, be noted that the change in heart rate is not marked, and in old animals may not be obtained at all.

TABLE 4. Isolated heart-lung-head preparation
Effect of occlusion of both carotid arteries

	Rate beats/min.	B.P. mm. Hg	V.P. mm. blood	Output c.c./min.
Normal	252	70	60	24·0
Occlusion	288	75	60	25·2
Normal	258	80	80	27·6
Occlusion	300	85	80	26·4
Normal	276	85	100	38·4
Occlusion	306	90	100	38·4
Both right and left vagus nerves cut				
Normal	330	60	60	24·0
Occlusion	330	65	60	24·0
Normal	330	68	80	26·4
Occlusion	330	68	80	26·4
Normal	330	85	100	36·0
Occlusion	330	85	100	36·0

These responses indicate that the small rapidly beating heart of the rat reacts in a manner similar to the much slower heart of the dog and they corroborate the accepted knowledge concerning the mammalian heart. In comparing the results of such work it should be noted that the venous pressure could be maintained constant irrespective of the rate of flow. In the experiments of Starling and his co-workers constancy of venous pressure depended on a determined rate of flow, the venous reservoir being placed well above the preparation and the blood flow controlled by a Hoffman clip. In the experiments on the rat the height of the venous reservoir has always determined the venous pressure.

Respiratory factors affecting cardiac output

Positive ventilation

The isolated heart-lung preparation. In experiments on the isolated heart-lung preparation it has invariably been the custom to inflate the lungs by positive ventilation.

It was thought that perhaps changes in the vascular bed of the lung occasioned by changes in the degree and frequency of such inflation would produce changes in the output of the heart. That changes in the rate of respiration, tidal air and pulmonary ventilation when produced by a positive ventilation pump, do not cause any changes in cardiac output is clearly shown by the data of numerous experiments. Table 5 gives in detail the data of one of these experiments. At a constant venous pressure and with heart rate unchanged in the denervated heart or the heart-lung preparation no changes in respiration rate or volume of tidal air (stroke volume of the pump) affect the constancy of the cardiac output. Not until the lungs are definitely damaged by excessive pulmonary ventilation does the output diminish.

TABLE 5. Isolated heart-lung preparation. Positive ventilation
Rat weight: 375 g. Heart weight: 1·0 g.
Effect of changes in respiration rate and pulmonary ventilation

Rate beats/min.	B.P. mm. Hg	V.P. mm. blood	Output c.c./min.	Respiration		Pulmonary ventilation c.c./min.
				Rate	Tidal air c.c.	
264	80	70	30·0	72	2	144
264	80	70	30·0	96	2	192
264	80	70	30·0	102	2	204
264	80	70	30·0	108	2	216
264	80	70	30·0	72	3	216
264	80	70	30·0	96	3	288
264	80	70	30·0	108	3	324
264	80	70	30·0	114	3	342
264	80	70	30·0	144	3	432
264	80	70	30·0	160	3	480
264	80	70	30·0	180	3	540
264	80	70	30·0	180	1	180
264	80	70	30·0	72	2	144

The isolated heart-lung-head preparation. When changes in positive pulmonary ventilation are made at different venous pressures the cardiac output varies as if only the venous pressures were altered. As has already been shown, increased venous pressure is associated with an increase in heart rate.

With moderate blood pressure, the changes in positive respiration, be it frequency, tidal air or pulmonary ventilation, have no effect on heart output. Minimal changes in heart rate, following increases in tidal air, are observed in lightly anaesthetized animals. Bainbridge [1920] stated that excessive artificial ventilation, by means of a pump, does not influence heart rate.

TABLE 6. Isolated heart-lung-head preparation. Positive ventilation
Rat weight: 350 g. Heart weight: 0·85 g.
Effect of changes in rate of respiration and in tidal air volumes at various venous pressures and moderate blood pressure

Rate beats/min.	B.P. mm. Hg	V.P. mm. blood	Output c.c./min.	Respiration Rate	Respiration Tidal air c.c.	Pulmonary ventilation c.c./min.
330	75	40	15·6	72	3	216
—	75	40	15·6	72	4	288
330	75	40	15·6	72	5	360
372	80	60	20·4	72	2	144
372	80	60	22·8	72	4	288
384	80	60	22·8	72	6	432
376	85	70	28·8	72	2	144
—	85	70	30·0	72	4	288
384	85	70	30·0	72	6	432
372	85	70	30·0	140	2	280
—	85	70	30·0	140	4	560
376	90	70	30·0	140	6	840
324	70	40	18·0	140	2	280
—	70	40	18·0	140	4	560
324	70	40	18·0	140	6	840
324	70	40	18·0	192	1	192
—	70	40	18·0	192	2	384
324	70	40	18·0	192	3	576
252	75	60	27·6	54	4	216
264	78	60	27·6	54	6	324
288	85	70	30·0	72	1	72
288	85	70	30·0	72	3	216
294	85	70	31·2	72	5	360
300	88	70	31·2	72	6	432
288	85	70	30·0	72	10	720
276	85	70	27·6	108	2	216
288	85	70	27·6	108	4	432

While in the isolated heart-lung-head preparation in the rat, an increase in tidal air volume causes a slight increase in heart rate, excessive positive ventilation results in a return of the heart rate to the normal level or below (Table 6). It would appear that the rate of respiration plays no part in the reflex, the extent of inflation being alone responsible. These results would corroborate those of Anrep, Pascual & Rössler [1936] on cardio-accelerator responses in the dog.

Negative ventilation

The isolated heart-lung preparation. In marked contrast to the lack of response of cardiac output to changes in positive ventilation are the results

TABLE 7. Isolated heart-lung preparation. Negative ventilation

Rat weight: 330 g. Heart weight: 0·8 g.

Effect of changes in rate of respiration and in tidal air volumes, venous pressure being constant

Rate beats/min.	B.P. mm. Hg	V.P. mm. blood	Output c.c./min.	Respiration		Pulmonary ventilation c.c./min.
				Rate	Tidal air c.c.	
318	85	70	21·6	48	1·2	58
318	90	70	26·4	48	2·0	96
318	98	70	33·6	48	3·7	180
318	105	70	36·0	48	6·0	288
318	92	70	27·6	72	1·2	86
318	98	70	32·4	72	2·7	198
318	100	70	34·8	72	3·2	230
318	105	70	37·2	72	4·2	302
318	105	70	38·4	72	5·2	374
318	90	70	26·4	90	1·2	112
318	92	70	28·8	90	2·0	180
318	95	70	31·2	90	2·6	234
318	100	70	34·8	90	3·0	270
318	105	70	37·2	90	4·0	360
318	85	70	24·0	120	0·8	96
318	90	70	26·4	120	1·0	120
318	95	70	31·2	120	2·4	288
318	98	70	34·8	120	3·3	396
318	100	70	37·2	120	4·2	502
318	85	70	24·0	150	1·0	150
318	90	70	27·6	150	1·5	225
318	95	70	31·2	150	2·4	360
318	98	70	33·6	150	3·0	450
318	100	70	34·8	150	4·0	600

arising from similar changes with negative ventilation. While, in the denervated heart, there is no change in rate, there is, with a constant venous pressure, a definite and progressive increase in the cardiac output with every

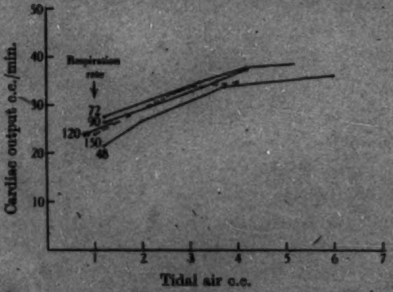

Fig. 6. Isolated heart-lung preparation with negative ventilation. Changes in the rate of respiration are without appreciable effect on the relationship of tidal air to cardiac output.

increase in negative ventilation. Associated with this increase in output is a small increase in blood pressure (Table 7). Examination of the details of the experiment shows that the increased output is a function of the increased venous pressure occasioned by a greater 'intrathoracic negative pressure'. The degree of increase in 'intrathoracic negative pressure' is indicated by the changes in tidal air. At constant tidal air intakes, the respiration rates, varying from a normal for the rat of 72 to 150/min., have no effect upon cardiac output. This is shown in Fig. 6, where, only when the respiration rate is reduced to the very low figure of 48/min., and where tidal air is small, is there an indication that increasing the rate to the normal figure may be slightly beneficial. This may be due to better oxygenation. Since the cardiac reflex pathways are not intact, all impulses of a reflex nature from the lungs or the heart are ruled out: the heart rate remains constant while cardiac output increases. This is the purely mechanical response to venous pressure obtained in all isolated heart-lung preparations and therefore it is apparent that increase in tidal air or in 'intrathoracic negative pressure' is responsible for the change.

Isolated heart-lung-head preparation. In this preparation results different from those described in the previous section have been obtained. When this preparation is negatively ventilated, alterations in tidal air volumes, at any maintained venous pressure, effect changes in both cardiac rate and output. As in the previous case, the 'intrathoracic negative pressure' which results from negative ventilation must be added to the venous pressure. The summation of these two effects is responsible for the increased venous pressure, which, causing a better filling of the heart and a greater tension in auricular muscle, leads to increased cardiac rate and output. It is apparent from Table 8 and Fig. 7 that changes in respiration rate, the venous pressure being constant, play no significant part in the changes effected. But the change in heart rate, which is clearly significant, remains the only vagal reflex that can be demonstrated under the conditions of these experiments. It has been shown that with negative ventilation, the vagi being cut, changes in cardiac output are determined by venous pressure and are independent of heart rate. With the vagi intact, an increase in the thoracic negative pressure increases cardiac output and at the same time there is a reflex increase in heart rate. The significance of the increase in thoracic negative pressure is that, by increasing the effective right auricular pressure, a marked increase in cardiac output is achieved with some conservation of the stroke volume of the heart.

Since alteration of tidal air volumes so affected heart output it was decided to see if this increase could play a part in restoring cardiac output under varying conditions of low venous and arterial blood pressures.

The part played by negative ventilation in the restoration of cardiac output. This was investigated under two conditions: (1) with low venous pressure (30 mm. blood) and optimal venous pressure (70 mm. blood), (2) with low arterial blood pressure (30–45 mm. Hg) and optimal arterial blood pressure (approx. 100 mm. Hg).

Table 8 and Fig. 7 show how the cardiac output at low venous pressures can be restored, if not to that obtaining at 70 mm. blood, at least closely

TABLE 8. Exp. 23. Isolated heart-lung-head preparation. Negative ventilation
Rat weight: 370 g. Heart weight: 1·1 g.
The effect of increasing 'intrathoracic negative pressure' on cardiac output at venous pressures 70 and 30 mm. blood and respiration rates 72 and 140/min.

				Respiration		
Rate beats/min.	B.P. mm. Hg	V.P. mm. blood	Output c.c./min.	Rate	Tidal air c.c.	Pulmonary ventilation c.c./min.
312	98	70	25·2	72	0·5	36
324	105	70	33·6	72	1·6	115
336	110	70	40·8	72	2·7	194
348	115	70	45·6	72	3·8	273
288	75	30	15·6	72	0·5	36
—	78	30	20·4	72	1·2	86
—	85	30	28·8	72	2·6	187
—	95	30	36·0	72	3·7	264
324	95	30	40·8	72	5·1	367
312	90	70	24·0	140	0·5	70
—	—	70	32·4	140	1·6	224
—	100	70	36·0	140	2·3	331
336	105	70	41·2	140	3·6	576
270	72	30	14·2	140	0·4	56
276	80	30	21·6	140	1·7	238
288	80	30	25·2	140	2·4	336
—	83	30	27·6	140	3·1	434
300	85	30	28·8	140	4·2	588

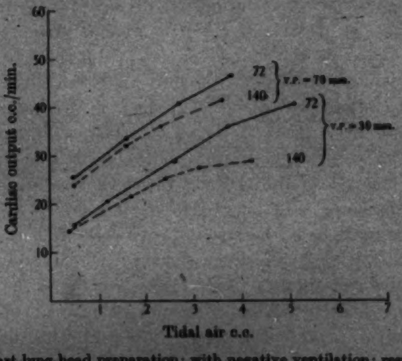

Fig. 7. Isolated heart-lung-head preparation; with negative ventilation; respiration rate 72/min. continuous line, 140/min. broken line. Increase in tidal air increases cardiac output at venous pressures of 70 and 30 mm. blood.

approximating it, by increasing pulmonary ventilation. With a venous pressure of 70 mm. blood and respiration rate 72/min., the cardiac output is 33·6 c.c./min. and the tidal air volume 1·6 c.c. At 2·7 c.c. tidal air volume, the output is 40·8 c.c./min., arterial blood pressure being 110 mm. Hg. This output, which can be regarded as optimal for the heart of a rat weighing between 300 and 400 g., is reached when, with a venous pressure of 30 mm. blood, the tidal air volume is 5·1 c.c. The arterial blood pressure, while not fully restored to 110 mm. Hg, is well maintained at 95 mm. Hg. The increased degree of expansion of the thorax is represented by the change in tidal air.

When the respiration rate is increased to 140/min. no great difference in cardiac output is obtained with a venous pressure of 70 mm. blood except that, when the tidal air volume exceeds 2 c.c., there is a slight fall in the rate of increase of cardiac output (Fig. 7). At a venous pressure of 30 mm. blood, however, an increase in tidal air up to 5 c.c. is not accompanied by a similar rise in cardiac output and arterial blood pressure.

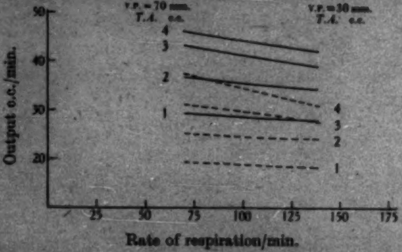

Fig. 8. Effect of rate of respiration on cardiac output at constant volumes of tidal air and with venous pressures of 70 and 30 mm. blood. V.P. venous pressure; *T.A.* tidal air in c.c.

The effect of rate (frequency) of respiration. That, in negative ventilation, the rate of respiration is not the determining factor in raising cardiac output is seen in Fig. 8 where are shown the data from Fig. 7 relating respiration rate to cardiac output at constant tidal air volumes, i.e. constant degrees of inspiration or constant 'intrathoracic negative pressures'. At a venous pressure of 70 mm. blood an increase in the rate of respiration from 72 to 140/min. has no significant effect on output until the tidal air volume exceeds 2·5 c.c., i.e. a low or moderate degree of inspiration, after which point it becomes detrimental to cardiac output. At a venous pressure of 30 mm. blood a similar situation arises. It would appear from such results that, with low venous pressure, increased 'intrathoracic negative pressure' is the effective means for increasing venous pressure and raising cardiac output, and that increased rate of respiration, while of no material benefit at low tidal air volumes, is to some extent harmful when tidal air volumes pass the normal amplitude of respiration.

The effect of low arterial resistance. Table 9 indicates the part played by 'intrathoracic negative pressure' in restoring cardiac output at low arterial

Table 9. Exp. 21. Isolated heart-lung-head preparation. Negative ventilation
Rat weight: 300 g. Heart weight: 0·8 g.
Effect of changes in venous and arterial blood pressure

Rate beats/min.	B.P. mm. Hg	V.P. mm. blood	Output c.c./min.	Respiration		Pulmonary ventilation c.c./min.
				Rate	Tidal air c.c.	
At optimal arterial blood pressure						
360	105	100	30·0	72	1·5	108
—	98	100	34·8	72	3·2	230
378	108	100	42·0	72	5·7	410
300	100	70	24·0	72	1·7	112
—	102	70	31·2	72	3·5	252
342	105	70	32·4	72	4·6	321
282	78	30	12·0	72	1·2	86
—	80	30	19·2	72	2·4	172
306	90	30	26·4	72	5·2	374
282	70	20	9·6	72	1·1	79
—	75	20	13·2	72	3·0	216
288	82	20	19·2	72	4·7	338
At low arterial blood pressure						
312	45	70	21·6	72	1·5	108
—	48	70	27·6	72	3·7	266
318	55	70	31·2	72	5·6	403
252	25	30	12·0	72	1·4	101
—	30	30	15·6	72	3·0	216
—	42	30	18·0	72	5·1	367
258	42	30	21·6	72	6·3	454

and venous blood pressures. The first part of the table shows the usual responses to change in venous pressure and tidal air volumes. The effects of low arterial blood pressure are shown in the second part of the table, where the pressure has been lowered by adjusting the peripheral resistance. With a venous pressure of 70 mm. blood, an arterial blood pressure reduced to 45 mm. Hg is improved to 55 mm. Hg as the 'intrathoracic negative pressure' is increased to give a tidal air volume of 5·6 c.c., and with this the cardiac output is increased from 21·6 to 31·2 c.c. which is equal to that obtained when the tidal air is 3·5 c.c., the venous pressure 70 mm. blood and the arterial pressure 102 mm. Hg. When, however, the arterial pressure is reduced to 25 mm. Hg by reducing the venous pressure to 30 mm. blood there is no such increase in cardiac output when the 'intrathoracic negative pressure' is 6·3 c.c. The arterial blood pressure is not thereby raised to an effectual level and ultimately falls. The heart invariably fails if measures taken to restore blood pressure by increasing venous pressure and cardiac output fail to raise the arterial blood pressure above 45 mm. Hg. The question naturally rises as to what may be regarded as the critical point in terms of arterial blood pressure, at which increases in 'intrathoracic negative pressure' will be of no avail in restoring and maintaining cardiac output. Arterial pressures below 45 mm. Hg, with the venous pressure at 70 mm. blood, may be partially restored, as in the experimental results shown in Table 9, by a marked increase in the 'intrathoracic negative pressure', but the output is never maintained for long periods, and with a low venous pressure, 30 or 20 mm. blood, no restoration of cardiac output to normal has ever been obtained. It is suggested therefore that an arterial pressure of 45 mm. Hg may be regarded as a critical pressure for the heart of the rat weighing 300 g.

Maintenance of the heart's action demands two things which cannot be dissociated one from the other: (1) a good venous pressure, and (2) a good arterial pressure. To give the heart something to pump is of no avail if the factors determining the coronary circulation are not effective. Such experimental results would suggest that there is a fundamental relation between cardiac output and the respiratory capacity of the chest, as well as a relation between cardiac output and heart rate on the one hand and the area of the venous channels at their entrance to or within the heart on the other. Research into the fundamental question of the relation of these factors to the metabolic activity of the mammalian heart and the body which contains it still remains to be done.

SUMMARY

1. Methods for making an isolated heart-lung and heart-lung-head preparation on the rat, and for the negative ventilation of these preparations are described.

2. The reflex responses of the rapidly beating heart of the rat are similar to those in larger animals. .

3. Changes in frequency and amplitude of positive ventilation of the lungs have no effect on cardiac output: on the other hand, with negative ventilation, when the tidal air volumes, which are indicative of changes in intrathoracic pressure, are increased, then the cardiac output is also increased. Frequency of respiration has no significant effect on cardiac output.

4. Low cardiac outputs, due to low venous pressure, can be restored to the optimum by increasing the 'intrathoracic negative pressure' provided the arterial blood pressure is sufficient to maintain a good coronary circulation.

5. An arterial blood pressure of 45 mm. Hg is regarded as critical for the continued activity of the heart of the rat. At pressures lower than this, and with low venous pressures, the heart rapidly fails. At this critical arterial pressure, and with a venous pressure of 30 mm. blood, no increase in 'intrathoracic negative presssure' will effect a permanent restoration of cardiac output and arterial blood pressure.

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FACILITATION, INHIBITION AND DEPRESSION AT THE 'ARTIFICIAL SYNAPSE' FORMED BY THE CUT END OF A MAMMALIAN NERVE

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In a recent paper, 'Fibre interaction in injured or compressed region of nerve', Granit, Leksell & Skoglund (1944) have given a brief account of the historical background of this problem and of their own finding, namely, that electrical stimulation of one of the motor roots of the sciatic nerve (cats) elicits a discharge in the corresponding sensory root to which it has been transmitted from an 'artificial synapse' formed by the cut end of the sciatic or the hamstring nerve. Several controls described in that paper enabled them to localize the point of transmission to this region and to exclude stimulus escape. A very much smaller discharge or none at all is transmitted in the opposite direction, sensory to motor. The unidirectional properties of the artificial synapse of the severed end of the nerve were ascribed to the lower rheobase and accommodation of mammalian sensory fibres as compared with motor fibres (Skoglund, 1942; Kugelberg, 1944; cf. for same result with frogs, Erlanger & Blair, 1936).

In this paper it will be shown that the artificial synapse has several properties of theoretical interest which are of importance for an understanding of synaptic activity in general; moreover, Granit *et al.* (1944) noted that the artificial synapse behaved like a real synapse in its dependence upon the degree of anaesthesia of the animal. If the cats were drugged so that reflex activity was depressed, then the artificial synapse also failed to transmit impulses. Nor did it work in asphyxiated nerves in which the spikes set up by an electrical stimulus were still practically normal. In fact, in order to act as an artificial synapse the cut end must be in very good condition. Decerebrate animals made good preparations unless they had lost too much blood; cats under 'dial' easily became too heavily narcotized. A chloralose narcosis (about 5 c.c./kg. of a 1% solution) was found to give very good enduring preparations.

TECHNIQUE AND PROCEDURE

Fig. 1 is a schematic illustration of the manner in which the experiment was set up. In most cases the stimulating electrodes (*St*) were on the motor root of L7, and the recording leads (*Re*) to the amplifier on its sensory root. In some cases, and, later on in the work, nearly always, the stimulating electrodes were on *both* L7 and S1 or on each branch of the divided L7. Thus local interference between successive stimuli could be avoided. All branches from the main sciatic stem were severed as far up as possible; all muscles around the spine and the hip were cut across, and the leg completely denervated. These precautions are very important (cf. Granit *et al.* 1944). For quantitative work it is best to cut the whole sciatic stem, including the hamstring, at the same level so as to have a single reflecting cross-section. The effects from two cross-sections can interfere and thus cause complications (Granit *et al.* 1944).

The stimuli were slowly repeated condenser shocks of less than 100μsec. duration. Two stimuli, conditioning shock plus test shock repeated as a group, have mostly been used. The conditioning stimulus has then been synchronized with the sweep circuit of the cathode-ray oscillograph so that, each time the beam of the cathode-ray passes horizontally across the face of the tube, this movement starts at the moment of stimulation of the nerve. Thus, when the film slowly passes vertically across the beam, a series of successive pictures of its horizontal movement are obtained upon which are superimposed (vertically) (i) the volley elicited by the first stimulus, (ii) the shock artefact of the second stimulus followed by (iii) its volley (see Figs. 7, 8).

The terms 'afferent' and 'efferent' will be used to refer to the regions of the artificial reflex are wherein impulses travel towards or away from the synapse, independently of whether sensory or motor nerves are involved.

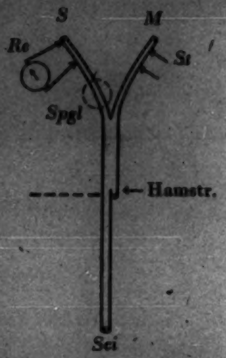

Fig. 1. Diagram illustrating stimulating electrodes (*St*) on motor root (*M*) and recording electrodes (*Re*) on sensory root (*S*). *Sci*, sciatic nerve; *Spgl*, spinal ganglion. In order to avoid interference between two reflecting cross-sections the whole nerve should be severed at the region marked by horizontal broken line.

RESULTS

1. *Two modes of action of the artificial synapse*

Rhythmic synapses. It was noted by Adrian (1930) that the 'injury' discharge from the cut peripheral end of a mammalian nerve is often rhythmically synchronized into beats. The rhythmic discharge gradually disappears. In Fig. 2*a* the discharge, picked up from the sensory root immediately after severance of the sciatic nerve, consists of synchronized beats at about 140 per sec. That these are coming from the cut end of the nerve is easily shown by several tests involving interference of some sort or other with that end. The records *a* show a set of successive pictures taken at the usual rate of 1–2 per sec. Upon these faster beats are superimposed slower rhythms which cause variations in their amplitude. The two upper records *b* were taken in succession, somewhat later after cutting the nerve, by which time the rhythmic discharge had diminished. Then a single stimulating shock to the (*St*) electrodes on the motor root was synchronized with the sweep so that in the two lower records *b* the shock initiates the sweep movement. The first effect of the shock to the motor root consisted in the appearance of a double volley (two peaks) in the sensory root. This first volley (cf. record) is preceded by a latent period, generally of the order of 3 msec. for nerves cut just above the knee. (For an explanation of the second peak see Granit *et al.* 1944.) The first volley is seen in the record to be succeeded by two large rhythmic beats. As a matter of fact a long train of such beats, gradually damping out, were started by the shock, as if it had operated some trigger mechanism at the artificial synapse, but, in the brief time during which the sweep traversed the face of the tube and was photographed, only two such beats had a chance to become included in the picture.

Now, in Fig. 2*b*, the last record directly succeeds the last but one (another stimulus has been applied) so that the rhythmic beating has been interrupted. It is interesting to note that the fresh *stimulus of the last record has reset the whole mechanism of excitation* and reiterated the events just described. These facts are typical, regular and easily repeatable, provided that the artificial synapse is of the kind in which the efferent (in this case = sensory) part of the reflex arc happens to be spontaneously beating. As previously pointed out by Adrian (1930) and Skoglund (1942) it is the rule to find the *sensory* fibres of the freshly severed mammalian sciatic discharging spontaneously, and quite often rhythmically. A new finding here is that activation of the artificial synapse from the *motor* end has such a remarkable influence on this kind of spontaneous activity. This raises questions as to the kind of changes of excitability which the shock has left behind in the synapse.

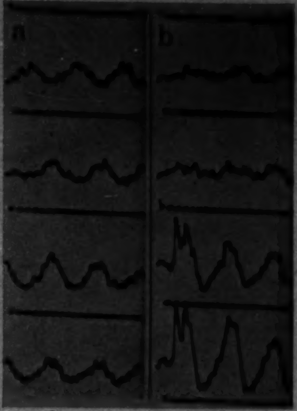

Fig. 2. *a*, spontaneous response from cut end recorded as indicated in Fig. 1. In the beginning of *b* the spontaneous oscillations have diminished but in the two last records of series *b* a shock to the motor L7, and synchronized with the beginning of the sweep to the extreme left, has activated the cut end to discharge a double-peak volley succeeded by a series of oscillations at the rate of the original rhythm. Time in msec. See text.

In the experiment of Fig. 2 the rhythm was obvious from the beginning; more commonly, however, and especially if the nerve had been severed for some 5 min. or more, there remained merely an irregular injury discharge. Despite absence of spontaneous rhythm the relayed response, caused by a shock to the motor L7 and recorded at the sensory L7 in the usual manner, consisted of two or three beats gradually damping out, the first volley always much larger than the secondary waves (see Figs. 7, 8). Finally, in some cases, when the 'reflex' response was as simple as the original motor volley from L7 and merely differed from it in being spread out over a longer duration, it was nevertheless possible to demonstrate by appropriate tests that periodic changes of excitability took place at the synapse. These will be discussed in the later sections of this paper.

The great majority of artificial synapses show some signs of rhythmic behaviour, gradually becoming less marked in an ageing preparation. But full rhythmic activity may then be restored by the simple expedient of making a fresh section farther up the nerve. For this reason it is suspected that non-rhythmic synapses are merely less active synapses. The easiest way of producing aperiodic synapses is by drugging the animals so deeply that general reflex irritability is depressed.

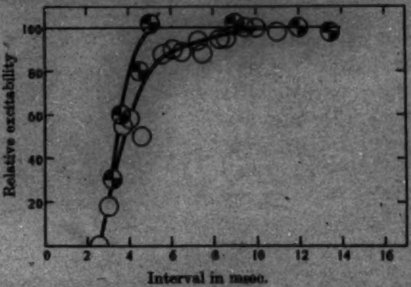

Fig. 3. Non-rhythmic artificial synapse. Abscissae: interval between conditioning shock and test shock in msec. Ordinates: relative excitability = size of second response expressed as percentage of normal control. Two different experiments. See text.

The non-rhythmic synapse. In the experiments of Fig. 3 two stimuli, conditioning stimulus and test stimulus, have been used. The test stimulus approaches the conditioning stimulus and consequently the relayed effect diminishes when the second stimulus falls during the state of refractoriness left by the first. This diminution is illustrated on a percentage basis against interval between the stimuli as abscissa. In the lower curve there is a slight indication of a hump at 5–6 msec. The upper curve is from another experiment with another animal. The refractoriness becomes 'absolute' at an interval between the stimuli of about 2·7 msec. The term 'absolute refractoriness', that here refers to the 'reflex arc', is used as a convenient shortening for 'least interval during which second stimulus is ineffective'. The two consecutive stimuli (causing maximal relayed waves) were in this case delivered to the same *St* electrodes but a great number of control experiments, demonstrating refractoriness for stimuli to different branches of L7, have shown beyond doubt that the seat of this long 'absolute refractory period' is the synapse (see below).

2. *The 'absolute refractoriness'. The 'synaptic delay'*

From thirty-seven measurements of the absolute refractoriness of the artificial reflex the statistical distribution curve of Fig. 4 was plotted. The extremes are 1 and 4·5 msec., the arithmetical mean 2·75. These measurements are from different experiments representing all kinds of artificial synapses: fresh sections, old sections, rhythmic and non-rhythmic ones, etc.

The synaptic delay was found by subtracting from the total latent period of the 'reflex' response the conduction times in the A group of motor and sensory fibres, as measured by a faster sweep. In such experiments it was found best to use the response relayed from the cut end of the hamstring nerve and keep the recording electrodes for measuring conduction velocity on the intact sciatic at the point where the hamstring was severed. Values of the order of 0·1–0·3 msec. were found for the synaptic delay. These are just at the limits of accuracy of the experiment, considering that the exact position of the synapse is unknown. It is concluded that the synaptic delay is so brief as to necessitate an electrical mechanism of excitation, comparable with the one responsible for the propagation of the action potential along the nerve. The same conclusion is suggested by Renshaw & Therman's (1941) interesting analysis of a response relayed into the sensory roots from a cross-section in the spinal cord.

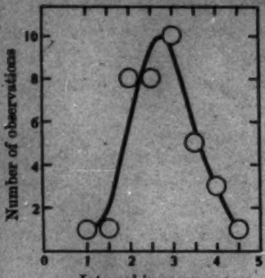

Fig. 4. Distribution of absolute refractoriness of artificial synapse over thirty-seven experiments. Ordinates: number of experiments at the different refractory periods grouped together for each 0·5 msec. Abscissae absolute refractory periods in msec.

3. *Rhythmic synapse and nerve compared*

The method of applying a test stimulus immediately after a conditioning stimulus has, of course, repeatedly been used with nerve (see especially Graham & Lorente de Nó, 1938, for work with blood-perfused mammalian nerve) and certain facts have become firmly established. Less is known about periodic changes of excitability following a conditioning shock and studied, e.g. by Erlanger & Blair (1936) and Lehmann (1937), though the available literature suggests that the rhythmic tendency is greatly favoured by diminished accommodation. v. Brücke, Early & Forbes (1941) speak of the recovery of 'responsiveness' when conditioning and test shock are strong; when the second shock is weaker the method measures 'excitability'. These terms will be used below in comparing a pure nerve experiment dealing with both 'responsiveness' and 'excitability' with a similar synapse experiment utilizing the same stimulating electrodes. In the nerve experiment the leads were at the cut end of the sciatic, in the synapse experiment on the sensory root L7 in the usual manner. The stimulating electrodes were on the motor L7.

Some records from the nerve experiment on *responsiveness* are found in Fig. 5 and the whole experiment is graphically summarized by curve 2 of Fig. 6. It gave for nerve the brief absolute refractory period of 0·4 msec. The second stimulus was uninfluenced by the first as long as the interval between the stimuli was greater than 2·5 msec. The recording electrodes were next shifted to the sensory root in order to repeat the same experiment with the artificial reflex. The stimuli were unchanged and just supramaximal. The relayed effects of conditioning and test stimulus alone are shown as 1 and 2 of Fig. 7. The interval between the stimuli was then systematically shortened, as shown in records 3–12 of the same figure. It is seen that, as the interval shortened, the second response passed through periodic maxima and minima of excitability. This experiment is reproduced as curve 1 of Fig. 6. At an interval of about 4 msec. the second volley actually was facilitated above its normal level, found in record 2.

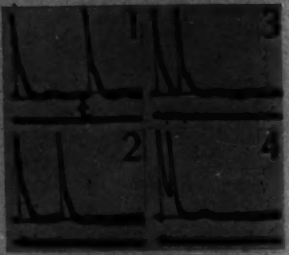

Fig. 5. Recovery of 'responsiveness' in motor L7. Conditioning stimulus, elicits sweep movement to the extreme left, test stimulus at the moment marked by small shock artefact (see arrow in record 1). Both stimuli supramaximal. The ensuing waves, as recorded at the cut end of the nerve (the 'synapse'), approach each other from 1 to 4. Recovery curve plotted as curve 2 of Fig. 6. Time in msec.

On returning the recording electrodes once more to the cut end of the nerve, but with the effect of the test shock diminished to 75% of the conditioning shock, the recovery curve numbered 3 of Fig. 6 was obtained. In this case excitability was measured, though merely graded in terms of 'size of the response relative to normal' in order to have the same ordinates as in the measurement of the 'reflex'.

Curve 3 should be compared with curves 1 and 2 in order to show that the introduction of an artificial synapse in the conducting nerve path, despite the use of supramaximal stimuli (curves 1 and 2), changes an experiment on responsivenss (curve 2) into one dealing with excitability. Curve 3 shows that similar curves are obtained with a pure nerve preparation by appropriate adjustment of the experimental conditions for determination of excitability. There is (in curve 3) the long 'least interval' between the stimuli, previously

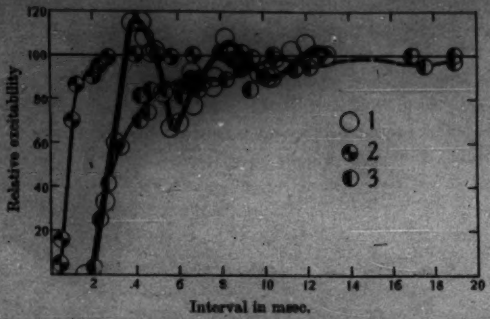

Fig. 6. Comparison of recovery in nerve and synapse. Abscissae: interval between conditioning shock and test shock in msec. Ordinates: relative size of test shock in percentage of normal control (line marked 100). 1, relayed response, recorded at sensory root; 2, direct response recorded at cut end, with supramaximal stimuli; 3, same, but with test shock 75% of conditioning shock. See text.

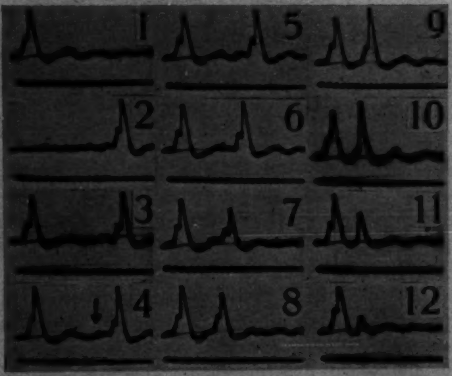

Fig. 7. Artificial synapse. 1, effect of conditioning stimulus alone; 2, test stimulus control, preceded by small artefact (see arrow in record 4); 3–12, shortening of interval between conditioning and test stimulus. Note, inhibition in 7, facilitation at shorter interval in 10, disappearance of secondary volleys during depression at still shorter interval in 12. The same results are plotted in Fig. 6, curve 1. Time in msec.

encountered as the 'absolute refractory period' of the 'reflex' arc. There are some oscillations of excitability (cf. also Graham & Lorente de Nó, 1938) though in this case, of course, located at the region under the stimulating electrode and not at the cut end. The changes of excitability in the artificial synapse similarly determine the number of fibres that is available for cross-excitation at the cut end.

4. *Depression at the synapse*

It is common to find that the states of absolute and relative refractoriness in the reflex arc are followed by a depression which by no means signifies a dying synapse. Fig. 8 illustrates an experiment of this kind.

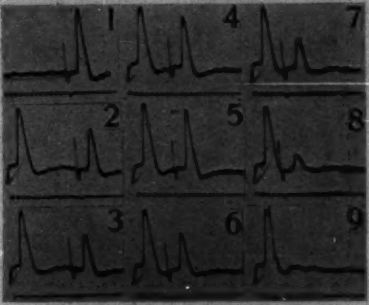

Fig. 8. Artificial synapse with marked depression. 1, test stimulus control preceded by large shock artefact; 2–9, shortening of interval between conditioning and test stimulus. Time in msec.

Record 1 is a control of the test stimulus alone. No increase of stimulus strength increased the relayed response which thus was maximal. In record 2 this stimulus was preceded by the conditioning stimulus. From 2 to 9 the interval was shortened. It is seen that throughout the illustrated intervals (from about 3 to 13 msec.) the test volley is below the level of its control (record 1) and thus depressed in size, even though within this depression there is a peak of relative facilitation in record 5, as compared with the records on either side of it.

In Fig. 9 some graphs of experiments with marked depression have been drawn for comparison. At the critical interval of 4–6 msec. there has been facilitation in the Exps. 1 and 2. In Exp. 2 the depression below the normal level of 100 lasted for about 32 msec., in Exp. 2 for 65 msec. In Exp. 3 of Fig. 9 the depression was unusually heavy. The test volley was at 40% of its normal size at an interval of 15 msec., and the depression lasted for 44 msec. There was no facilitation so that the synapse may be said to have belonged to the non-rhythmic type. But in the beginning of the experiment, as long as the section was fresh, there had been an initial facilitatory peak around 4 msec. Curve 3 is of especial interest also because in this case the stimulating electrodes were on different motor roots, the conditioning shock being delivered to L7, the test shock to S1. The recording leads were on the sensory L7 in the usual manner.

It is concluded from these facts that neither the early facilitation nor the depression require that the impulse volleys should pass along identical 'afferent' fibres. The stimulating volleys for the synapse can also 'converge' towards the cut end in different fibres. In several experiments 'converging afferents' were used in order to exclude processes under the electrodes.

Depression is certainly common in ageing synapses but this is not the whole explanation of the phenomenon. It must also depend upon other factors as yet unknown.

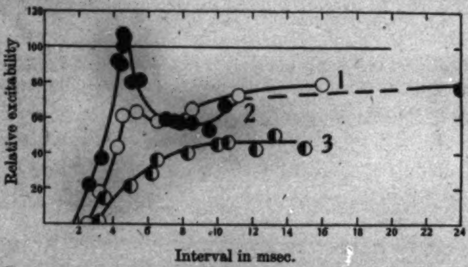

Fig. 9. Measurements of excitability in three different artificial synapses with marked depression. Abscissae: interval between conditioning and test shock as in Fig. 6. See text.

There is evidence to show that the states of depression are additive. Thus depression can easily be augmented by repetition of the stimulation at faster rates than 1–2 per sec., the rates which were used in most experiments. If the relayed volleys consisted of primary and secondary waves (Fig. 7) those with the longest latent periods disappeared first so that, under the influence of a gradually increasing frequency of stimulation, the relayed discharge was cut down from its tail end. At higher frequencies the reflex discharge was completely blocked at a frequency which did not very much affect the primary 'afferent' volley conducted to the synapse.

5. *Some properties of the periodic changes of excitability*

In some experiments the periodic changes of excitability of the artificial synapse have been followed for several periods. This was the case in the experiment of Fig. 10. The lower curve illustrates a combination of depression with the characteristic periodic facilitation and inhibition superimposed. The rhythm is gradually damping out in the typical manner. The upper curve 2 is from the same experiment but the test shock has now been made somewhat weaker so that the reflex response elicited by this shock was 84% of the one caused by the conditioning shock. This increased the crests of facilitation and the troughs of inhibition. Now a synapse is a gradually changing focus of cross-excitation so that comparative experiments of this kind cannot refer to exactly identical conditions, for it takes some time to complete a set of observations. However, this gradual change is in the direction of a diminution of amplitude of all periodic variations and an augmentation of depression. In this case, however, the experiment with the weaker test shock (curve 2) succeeded the one with less facilitation and more depression (curve 1). It is therefore held to be fully reliable and probably representing a phenomenon, which in the terminology of Sherrington and his collaborators (Creed, Denny Brown, Eccles & Sherrington, 1932), may be called 'occlusion'. It means that with maximal relayed volleys (curve 1) so many fibres of the 'neurone pool' have been engaged by the test volley that a smaller margin of fibres is left for facilitation. If the test shock is weakened the changes of excitability at the synapse have a greater chance of adding to or subtracting

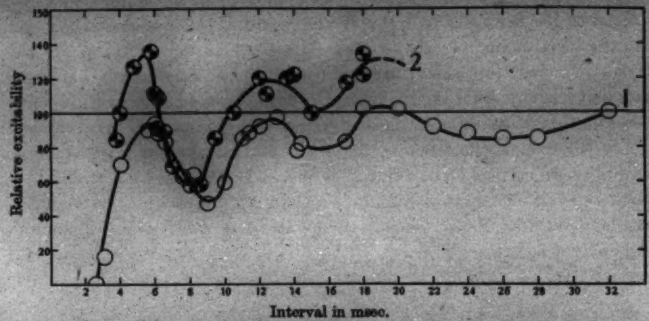

Fig. 10. Measurements of excitability in rhythmic artificial synapse with the method of conditioning shock and test shock as in Fig. 6. Fully explained in text.

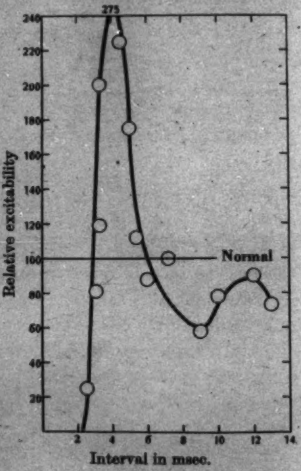

Fig. 11. Peak of increased excitability after conditioning shock in a single experiment. See text.

from the artificial reflex. Very weak test shocks have not been used on account of the irregularities that tend to complicate such measurements.

The greatest facilitation ever seen in one of these experiments is illustrated in Fig. 11. It reached 275% of the normal level of the isolated control. There, as in so many other cases, the peak of facilitation was exceedingly sharp. It is seen at an interval of about 4–5 msec. in Fig. 11.

The first facilitation maximum is the most constant in these experiments. It is generally at some interval between 4 and 6 msec. The second varies from 8 to 12 msec., and the third fluctuates still more. In some good experiments there have been three peaks of facilitation at 4, 8 and 12 msec. Between them there have been the typical troughs of inhibition. This corresponds to a frequency of 250 oscillations per sec. As pointed out above, the fluctuations of excitability are often large enough for the conditioning shock alone to cause a similarly fluctuating discharge to emanate from the synapse, and in many nerves the fresh cross-section discharges spontaneously at the same rate for several seconds, in some even for minutes. The maximal frequencies observed for the fluctuations in excitability have been around 300 per sec. It is impossible to note such facts without questioning whether or not the normal repetitive discharge of neurones is an expression of similar rhythmic fluctuations.

6. *Overlapping shocks to different roots*

It was expected that the use of different adjacent roots, or division of L7 into two halves, for conditioning and test shocks would make it possible to have the two shocks approaching each other with only moderate signs of interference, but this was not found to be the case. On the contrary, the results in this arrangement differed relatively little from those obtained when the shocks were delivered to the same electrodes. Both general depression and the periodic changes in excitability were again found (cf. § 4). Depending upon the preparation used and upon stimulus strength, the refractoriness became 'absolute' or merely 'relative' at short intervals. Precise summation of the reflex volleys was seen for coinciding stimuli but never a 'reflex' overshooting the sum of the volleys caused by conditioning shock and test shock controls. It is possible that facilitation at coincidence would have been obtained if weak stimuli had been used. However, since the artificial reflex was unstable with weak stimuli, all experiments in which the effects from an undivided root were compared with those, obtained when its components were separated into two halves, were carried out with strong stimuli.

Especially interesting is the experiment illustrated in Fig. 12. The stimuli were applied to the motor L7 and S1, and the relayed response was recorded from the sensory L7. The reflex effects were maximal but this meant that S1 elicited a response 67% of that initiated from L7. To the left of zero in the diagram, L7 received the conditioning shock, to the right of it the stimuli had overlapped and S1 received the conditioning shock.

The following points should be noted: whichever shock went first, it always left a period of absolute refractoriness for the other, as shown by the figure to either side of zero. Both curves indicate a generalized depression, which was most evident with S 1 leading; with 'L7 leading' the depression was interrupted by a fairly large peak of facilitation. At coincidence the relayed volley had the size of the larger response (from L7). They did not sum.

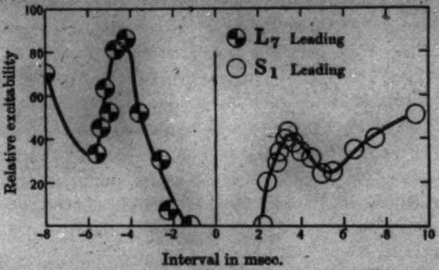

Fig. 12. Effect of successive stimuli applied to L7 and S1 on the excitability of the synapse. See text.

The experiment raises the question: how could S1, which only activated 67% of the number of fibres engaged by L7, leave the reflex path absolutely refractory for L7. The answer can only be that S1 has caused such a large generalized depression in the synapse, that the remaining 33% of the fibres of the 'final common path' were also affected. The peak of facilitation suggests that the two reflexes have had a final common path, but, as long as the fibres have not been identified and the mechanism of excitation is not fully understood, it is also best not to overemphasize the parallels with well-known results from the work on the spinal cord. How depression spreads in the artificial synapse is as yet unknown.

Discussion

When Hering (1882) first found cross-excitation at a cut end of the sciatic plexus of frogs, it had the character of an exceptional phenomenon, present only in animals showing signs of increased irritability. In cats this situation appears to be the normal state of affairs, probably on account of the low accommodation of the sensory fibres in this animal (Skoglund, 1942). Jasper & Monnier (1938) found cross-excitation in crab nerve, and Arvanitaki (1940 *b*, *c*) showed that the impulse passed across two parallel, adjacent and partly overlapping crab nerves, provided that the 'efferent' contact point of the artificial synapse had been treated with citrate. From this work one might conclude—and it seems to have been concluded—that cross-excitation easily takes place in crab nerves. Later, however, Katz & Schmitt (1940) have shown that in such nerves also there is a large safety margin provided for isolated conduction. This paper and that of Granit *et al.* (1944) emphasize that the correct explanation of the results of Jasper & Monnier must be that in their cases too the effect was localized to the cut ends of the nerves tested. This would explain their long 'synaptic delay'.

In her crab nerve synapse, treated with citrate, Arvanitaki (1940 *a*, *c*) has found a series of oscillating 'local potentials'. The first effect of the 'efferent' impulse is a negative 'local response', called the 'pre-potential', identical with the local response of Arvanitaki (1936) and Hodgkin (1938). In synapses of the rhythmic type it is succeeded by further oscillations, 'consecutive potentials', gradually damping out. The impulses are generated on top of these local changes. With leads at the artificial synapse, formed by the cut end of the cat sciatic, we have also found similar oscillating potentials which in some cases have not been conducted away to the sensory root. The excitability changes have varied in parallel with those potentials. We hope to return to this aspect of the problem with a detailed investigation of the phenomena at the synapse itself.

It would seem to be justifiable to regard the processes at the artificial synapse as something not very far removed from probable events at a real synapse. Bernhard (1944) has recently studied the interaction of a conditioning stimulus to the popliteal nerve with a test stimulus placed in the spinal cord itself (cats). The effect was recorded in the ipsilateral peroneal nerve. The stimulus to the popliteal nerve was found to be succeeded by a fast periodic variation of excitability strongly reminiscent of the effects described in this paper. Quite often a single shock to the spinal cord is seen to elicit a series of rhythmic oscillations of grouped impulses in the efferent nerve (cf. Lloyd, 1941). Bernhard & Granit (1942) have shown that this complex relayed response is cut down from the tail end when stimulus frequency is increased, just as is the case with the volleys relayed from the artificial synapse under similar circumstances.

To sum up, without stressing the analogies, we may say that the work with artificial synapses in peripheral nerve provides an encouraging approach to the physiology of the central nervous system.

SUMMARY

1. The cut end of the cat's sciatic nerve forms an artificial synapse in which a volley passing in the motor fibres is relayed across to the sensory fibres. The effect, established by Granit *et al.* (1944), is here analysed from

the point of view of the changes of excitability which transmission of a volley across the 'synapse' elicits in the latter.

2. Most artificial synapses respond to a single shock to the motor roots by setting up *a periodic variation of excitability*, always preceded by a state of refractoriness. The periodic variation in excitability and the preceding refractoriness have been analysed with the aid of a second test shock. The relayed response of the test shock is *facilitated or inhibited*, depending upon whether it falls in the crest or trough of the periodic change of excitability left by the conditioning shock. It is immaterial whether the two shocks are given to the same root, to separate branches of the same root, or to adjacent roots sending fibres to the cut end which is acting as synapse.

3. In some synapses the periodic changes are superimposed upon a *generalized depression of excitability*, left by the conditioning shock.

4. The artificial synapse does not transmit impulses unless the anaesthesia is light and the animal is reflexly active.

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THE MECHANISM OF WATER DIURESIS IN NORMAL RATS AND RABBITS AS ANALYSED BY INULIN AND DIODONE CLEARANCES

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Renal clearances of inulin and diodone, and hence certain important aspects of renal function, have hitherto only been incompletely studied in small laboratory animals. Kaplan & Smith (1935) estimated inulin clearances in rabbits and found that, in contrast to certain other mammalian species like the dog and human adults, inulin clearances increased with increasing urine flow. Diodone clearances in rabbits do not seem to have been recorded. Determination of inulin and diodone clearances in rats have recently been reported (Friedman & Livingstone, 1942). However, there are several features of the experimental technique used by these investigators which would seem to limit the usefulness of their results. (1) The urine samples were obtained from anaesthetized animals (type of anaesthetic not stated). (2) Owing probably to the use of an anaesthetic, the urine flow of their animals was uniformly very low (M. = 0·0033 ml./100 g./min., S.D. = ± 0·00157) and this in spite of the fact that a saline diuretic (2 % sodium sulphate) had been administered some time before the experiment started. (3) Friedman & Livingstone's results, based on experiments on animals with a very limited range of urine flow, do not make it clear whether inulin clearances, and thus glomerular filtration rates, of rats change with alterations in urine flow. That is to say, it is not possible to see from their experiments whether, with reference to this important feature, renal function in the rat conforms to the 'rabbit type' or to the 'human type' of kidney.

It is clear that the interrelation between inulin and diodone clearances and rate of urine flow had to be ascertained if, as in the present case, clearance determinations on normal rats and rabbits were meant to precede work which would involve experimental changes of renal function. A study of the renal mechanism of water diuresis was implicit in such an investigation.

METHODS

Experimental animals. Thirty-five male adult albino rats and eight rabbits (7 ♂, 1 ♀) were used.

Feeding. The rats were given about 15 g. per day of Vitamin A Test Diet (U.S. Pharmacopoeia XI, revised 1937) with the addition of cod-liver oil and tocopherol. The animals received this diet for at least 2 weeks before experiments started and then throughout the period of experimentation. The average weight of the rats, before experiments started, was 181·3 g. An average increase of body weight of 57·5 g. after 3 months suggested that the experimental procedures performed during that period did not interfere with the health of these animals.

The rabbits received oats and water and oats and greens on alternate days.

Experimental procedures for the determination of simultaneous inulin and diodone clearances. A. *Rats.* Inulin and diodone were injected subcutaneously as one injection. This mode of administration has recently been used with satisfactory results by several investigators (Findley & White, 1940; Heinbecker, Rolf & White, 1943; Bobey, Longley, Dickes, Price & Hayman, 1943). The doses per animal ranged from 2·0 to 4·0 ml. of a 5 % solution of inulin in physiological saline and from 0·3 to 0·8 ml. of a 35 % solution of diodone. The amounts injected varied according to the approximate serum level desired.

The routine procedure for determining the clearances was as follows: Thirty minutes after the injection of inulin and diodone the rats received 5 % of their body weight of tepid water by stomach tube. The administration of this fairly large volume of fluid did not only have the advantage of yielding an extensive range of urinary excretion rates, but enabled short periods to be used for the collection of urine. Sixty minutes after the injection the animals' bladders were emptied. Handling or prodding proved an effective stimulus for micturition, but the application of suprapubic pressure was used as a measure of control. Each rat was then placed in a specially devised small metabolism cage. A floor space diameter of 5 in., and spacing of the floor wire at 0·5 in., ensured minimal losses of urine. At about 75 min. the bladder was again emptied, using the same method as before, and the urine carefully collected and measured. The time from the placing of the animal in the metabolism cage to the time of obtaining the last drops of urine, the 'urine collecting period', varied from 10 to 25 min., according to the urine flow of the animal. Individual urine collecting periods were timed with the utmost accuracy attainable.

Immediately after the end of the urine collecting period the animals were anaesthetized and blood taken from the tail. The amount of blood taken for each clearance experiment did not exceed 2·5 ml. The anaesthetic used initially was pentobarbital sodium (0·45 ml. of a 0·8 % solution per 100 g. rat) but light ether anaesthesia was later found to be more suitable. The short duration of our urine collecting period enabled us to dispense with a 'mid-period' blood collection. Anaesthesia in rats during the urine collecting period would have produced an inhibition of diuresis (Heller & Smirk, 1932) which, in turn, would have forced us to extend the period of urine collection to a length which would have deprived a 'mid-period' blood collection of its theoretical advantage. The possibility of a pharmacological effect of the anaesthetic on renal function was a further factor which rendered a mid-period blood collection inadvisable. Such a pharmacological effect, leading to abnormal clearance values, has recently been noted by Corcoran & Page (1943), following the use of pentobarbital sodium in the dog.

Up to five simultaneous clearance determinations of inulin and diodone were performed on one rat at intervals of four weeks. In order to determine whether the repeated bleeding produced any permanent dilution of the blood, a series of haematocrit estimations were performed at suitable intervals. No significant changes were found in rats which had been bled repeatedly. It is likely, therefore, that as far as the physical properties of the blood were concerned, the clearance determinations on the same animal were done under comparable conditions.

B. *Rabbits.* Inulin and diodone were administered as one subcutaneous injection. The doses per animal ranged from 5 to 20 ml. of a 10 % solution of inulin and from 2 to 5 ml. of a 35 % solution of diodone. The following was the routine procedure used: Two hours after the injection of inulin and diodone the animal was given 5 % of its body weight of tepid water by stomach tube. The same dose of water was again given 1 hr. later. One hour after the second administration of water the bladder was emptied by suprapubic pressure; the animal was then placed in a metabolism cage and the urine collecting period started. The duration of the urine collecting periods varied from 10 to 25 min., according to the rate of urine flow. Individual urine collecting periods were timed accurately. Blood samples from the marginal ear vein were obtained immediately after the end of the urine collecting period. Several clearance determinations were usually performed on the same animal during the same day. A third dose of water was sometimes given after the first collection of blood.

Analytical methods. Inulin in serum and urine was determined by the method of Smith, Goldring & Chasis (1938). By this method inulin has a glucose equivalent of about 100 % and all figures are reported as apparent glucose. Diodone iodine in serum and urine was determined by Alpert's (1941) method. White & Rolf's (1940) method was tried but was found to be less accurate. A series of control experiments showed that there was no essential difference between the results of inulin and diodone estimations in serum or in plasma.

The inulin and diodone preparations used were Inulin (Kerfoot and Co.) and Per-Abrodil (Bayer Products Ltd.).

Inulin and diodone clearances (C_{IN} and C_D) have been expressed as ml./100 g. body weight/min. Considering that surface determinations would have been based on body weight, it was felt that no advantage would have been gained by relating clearances to body surface. Definitions and methods of calculation of the other renal functions analysed conform to those of Smith and his co-workers.

The following formula was used to calculate the rate of tubular excretion of diodone (T_D):

$$T_D=\left(\frac{C_D}{C_{IN}}-WF\right)DC_{IN},$$

where D=amount of diodone iodine per ml. of plasma, $W=1{\cdot}00-\frac{\%\text{ plasma solids}}{100}$, F=plasma diodone iodine/ultrafiltrable diodone iodine ratio. The values for W and F of rat plasma were taken from the paper of Friedman & Livingstone (1942). The values for W and F of rabbit plasma were determined by the following methods:

Determination of W. Samples of heparin plasma were obtained from five animals. The plasma solids were estimated by drying at 103° C. Duplicate determinations gave 7·50±0·031 % as the mean value of the plasma solids of the five samples. W, calculated according to the above formula, was therefore 0·925.

Determination of F. About 10 ml. of blood from the marginal ear vein of an unanaesthetized rabbit, which had been injected with diodone, were collected under paraffin in a centrifuge tube containing heparin and immediately centrifuged. A part of the plasma was passed through a Bechold (glacial acetic acid-pyroxylin) ultrafilter. Diodone iodine was estimated both in the plasma and in the ultrafiltrate: the plasma iodine/ultrafiltrate iodine ratio was then calculated. Such determinations were made on plasma samples of six animals and gave 0·70±0·017 (S.E. of mean of six observations) as the mean value of the ratio. The mean value of F adopted for use in the formula for T_D was 0·7.

RESULTS

Rats. 104 simultaneous clearance determinations of inulin and diodone were performed on 35 rats. A survey of the values obtained for inulin clearances showed that they remained approximately constant with variations of serum inulin concentrations ranging from 5 to 314 mg./100 ml. serum. To substantiate this statement the clearances were expressed as a mean and standard error for three groups, those with low (5–20 mg./100 ml.), medium (21–60 mg./100 ml.) and high (61–314 mg./100 ml.) serum inulin concentrations. The mean clearance of the first group was 0·365 ± 0·0188 (18) ml./100 g./min., that of the second group 0·357 ± 0·0097 (54) ml./100 g./min. and that of the third group $0{\cdot}337 \pm 0{\cdot}0138$ (32) ml./100 g./min. A comparison of these means yielded the following figures for t and P (see Fisher & Yates, 1943): a comparison of the means of the first and second group gave $t = 0{\cdot}408$, $P < 0{\cdot}7 > 0{\cdot}6$, a comparison of the means of the second and third group $t = 1{\cdot}220$, $P < 0{\cdot}3 > 0{\cdot}2$ and a comparison of the means of the first and third group $t = 1{\cdot}292$, $P < 0{\cdot}3 > 0{\cdot}2$. It will be seen that none of the values of t are significant. There is therefore no evidence for a significant difference between the mean clearances of the three groups.

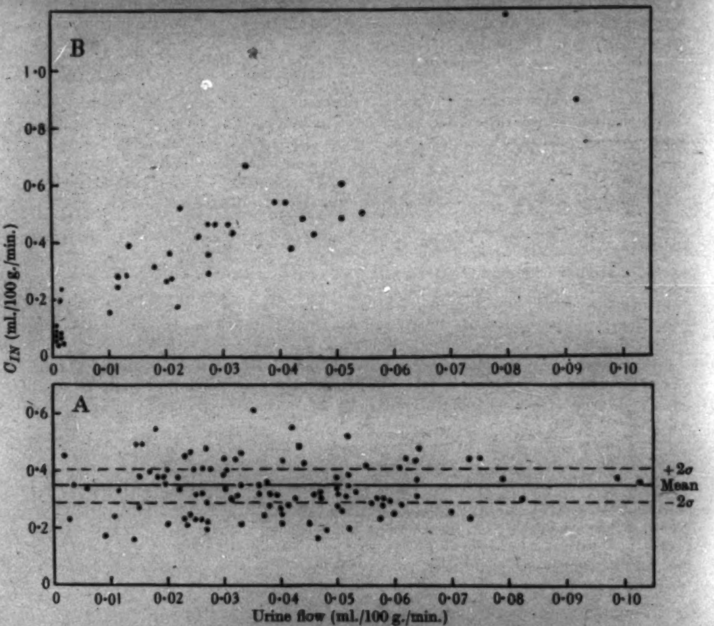

Fig. 1. A, normal adult rats; B, normal adult rabbits. Inulin clearance (C_{IN}) in relation to rate of urine flow.

The survey of the values for inulin clearances showed further that they remained approximately constant with variations of urine flow ranging from 0·0035 to 0·1030 ml./100 g./min. (Fig. 1A). In other words, the glomerular filtration rate stayed practically constant even at the very low and the very high rates of urine formation encountered in our experiments. The mean value of C_{IN} obtained from all the experiments of these series was $0{\cdot}351 \pm 0{\cdot}0027$ (104) ml./100 g./min.

Diodone clearances in rats were determined at blood iodine levels ranging from 0·58 to 164·97 mg. I/100 ml., and variations of urine flow ranging from 0·0035 to 0·1030 ml./100 g./min. Fig. 2 shows that, as in man or the dog, (*a*) high clearance values were obtained at low blood diodone levels, (*b*) the diodone clearances decreased with a rise in the blood diodone level, and (*c*) as a consequence of the constant glomerular filtration rate (Fig. 1 A) C_{IN}/C_D increased with the depression of the diodone clearance. The first point on the C_D curve representing the mean of the highest diodone clearances obtained in our series, may be regarded as a measure of the renal plasma flow in normal adult rats.

There appeared to be no significant change of the diodone clearance with an increase in urine flow. This could be demonstrated in the following manner: C_D values obtained at low blood diodone levels (0·58–4·68 mg. I/100 ml. serum) were selected and divided into two groups which differed in the rate of urine flow at which C_D had been estimated. The first group comprising urinary excretion rates ranging from 0·0140 to 0·0305 ml./100 g./min. gave a mean C_D of 1·77 ± 0·211 (8) ml./100 g./min. The second group comprising urinary excretion rates ranging from 0·0320 to 0·0666 ml./100 g./min. gave a mean C_D of 2·09 ± 0·178 (10) ml./100 g./min. A comparison of these means gave $t = 1{\cdot}171$ and $P < 0{\cdot}3 > 0{\cdot}2$. In other words, the difference between the means was statistically not significant. The means of the blood diodone concentrations (in terms of diodone iodine) at which the C_D values had been obtained were 3·00 ± 0·399 (8) mg. I/100 ml. for the first group and 2·43 ± 0·415 (10) mg. I/100 ml. for the second group. The difference between these means was not significant ($t = 0{\cdot}972$, $P < 0{\cdot}4 > 0{\cdot}3$) showing that the two groups came from the same population. The conclusion that the diodone clearances in the rat which were obtained at low blood diodone levels did not increase with an increase of urine flow seems therefore justified.

In order to facilitate the estimation of the maximal rate of tubular excretion of diodone (Tm_D) from our data, the T_D values were divided into classes and the mean of each class calculated. It will be seen from Fig. 2 that the blood iodine level of classes I–IV rises by 2 mg./100 ml. serum, that of classes V and VI by 4 mg./100 ml. serum and that of class VII by 8 mg./100 ml. serum; experiments done at still higher blood iodine levels are not represented on the figure. The progressive depression of the mean clearance values with the rise of the blood diodone content is clearly discernible. It will also be seen that the mean T_D value of the first four classes increased proportionately to the rise of the blood diodone content. However, once the diodone iodine level had reached approximately 12 mg./100 ml. serum a rise of the T_D value was no longer observed. This maximal value of T_D should therefore be the expression for the tubular excretory mass of the rat kidney. Tm_D as derived from classes V, VI and VII of our series of experiments amounted to 0·1257 ± 0·00269 mg. I/100 g./min.

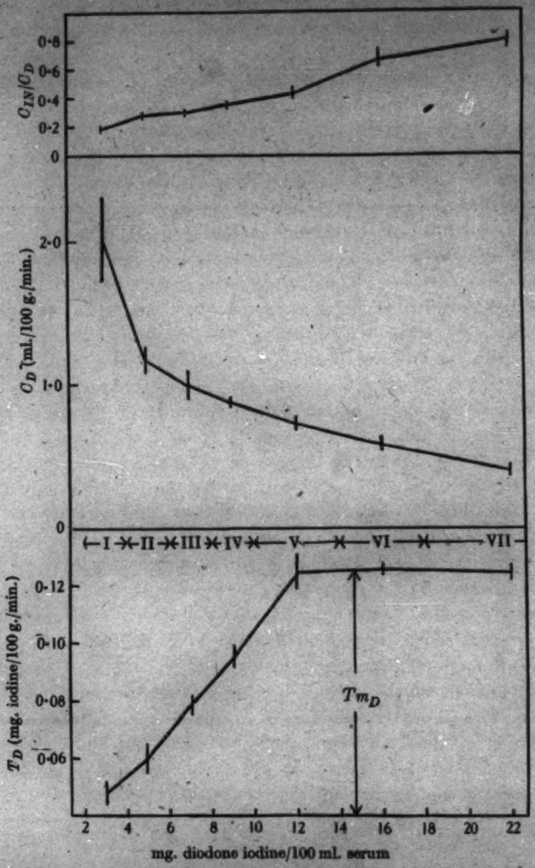

Fig. 2. Normal adult rats. The influence of the blood diodone level (in terms of serum iodine) on diodone clearance (C_D), rate of tubular excretion of diodone (T_D) and C_{IN}/C_D ratio. The vertical lines indicate the standard error. The roman numerals between horizontal arrows indicate the 'classes' into which the data were divided in order to obtain mean values. The serum iodine concentration of classes I–IV rises by 2 mg./100 ml., that of classes V and VI by 4 mg./100 ml. and that of class VII by 8 mg./100 ml. The experiments on which these curves are based were performed within the same range of urine flow as the inulin clearances of Fig. 1A.

Rabbits. Sixty-four simultaneous determinations of inulin and diodone clearances were performed on eight rabbits. Fig. 1B shows, in confirmation of the findings of Kaplan & Smith (1935), that the inulin clearances in the rabbit increased with increasing urine flow. A difference between the results of Kaplan & Smith and the results obtained in this investigation consists in the more extended range of the urinary excretion rates at which inulin clearances were obtained. In contrast to Kaplan & Smith no difficulties were experienced in achieving very high diureses in rabbits. In no case were convulsions or death due to water intoxication observed. The highest rate of urine flow observed by Kaplan & Smith was 5·60 ml./sq.m./min., whereas values up to 16·06 ml./sq.m./min. (corresponding to 0·093 ml./100 g./min.) were reached in the present experiments. An explanation for this discrepancy is perhaps afforded by a different nutritional regime and by the different time interval between the administrations of water.

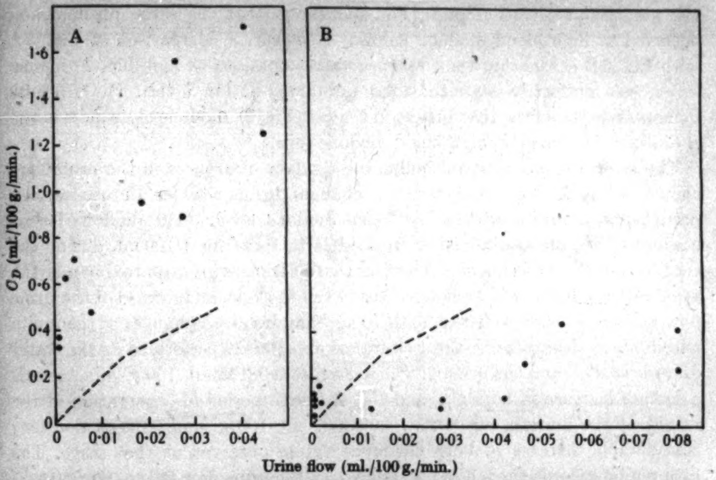

Fig. 3. Diodone clearances of normal adult rabbits in relation to glomerular filtration rate and urine flow. A, diodone clearances obtained at low blood diodone levels (range: 1·48 to 2·95 mg. diodone iodine/100 ml. serum) showing the increase of the diodone clearance with the rise of the glomerular filtration rate (broken line). B, diodone clearances obtained at high blood diodone levels (range 20·8–47·8 mg. diodone iodine/100 ml. serum) showing the depression regularly observed at high blood diodone levels. Note, however, that even the depressed clearances follow to some degree the rise of the glomerular filtration rate. The broken line (=glomerular filtration rate) was obtained from the data recorded on Fig. 1B by grouping and averaging the values falling within a rise of 0·01 ml./100 g./min. of urine flow.

The determination of C_D in the rabbit is clearly more involved than the determination of this value in the rat: the changes of the glomerular filtration rates in the rabbit with variations of urine flow introduce a new variable. It was therefore necessary to consider diodone clearances in animals with (*a*) low and (*b*) high blood diodone levels at increasing rates of urine flow.

Considering first the trend of the diodone clearance at low blood diodone iodine values, it will be seen from Fig. 3A that C_D rose with the increase of the glomerular filtration rate. Fig. 3B shows that the same phenomenon occurred at high blood diodone values. However, a comparison of Fig. 3A with Fig. 3B shows that the clearance values obtained at high blood diodone levels were uniformly lower than those obtained at low levels. These results demonstrate therefore, that just as in the rat (Fig. 2), diodone clearances in the rabbit are depressed at high blood diodone levels.

The interrelations between inulin and diodone clearances in the rabbit are shown in Fig. 4. Fig. 4A shows renal changes during a water diuresis as they occurred in a rabbit with a low blood diodone level. (The diodone iodine content of the blood diminished from 4·024 to 1·480 mg. I/100 ml. during the six hours of the experiment.) The first two clearances give approximately the same picture, but it will be noticed that even so slight an increase of the urine flow as from 0·00046 to 0·00055 ml./100 g./min. involved changes of the inulin and diodone clearances. A third clearance experiment performed as the water diuresis started and the urine flow increased to 0·00178 ml./100 g./min. reveals a further increase in both C_{IN} and C_D. A fourth period of observation at the height of the diuresis (urine flow=0·02600 ml./100 g./min.) shows the very considerable increase of both clearance values observed at that stage. The fifth period records the fall of C_{IN} and C_D as the urine flow fell to 0·01930 ml./100 g./min. The parallelism in the change of urine flow, glomerular filtration rate and diodone clearance in Fig. 4A will be noted, in contrast to the results in rats in which C_{IN} and C_D remained essentially unchanged during the rise and fall of a water diuresis. Fig. 4B shows an experiment in which the blood diodone was maintained at a high level (decrease of blood iodine from 30·477 to 17·978 mg. I/100 ml. in 5 hr.). A parallel rise in urine flow and glomerular filtration rate similar to that shown in Fig. 4A was observed. The values for C_D show the depression due to the high blood diodone level and it will be seen that they changed little during the first three periods of observation. However, the pronounced rise of the glomerular filtration rate observed during the fourth period coincided with a definite increase of the depressed clearance.

T_D values were found to increase with an increase of the glomerular filtration rate both at low and at high blood diodone levels (Figs. 4A and 4B). The significance of the changes of the C_{IN}/C_D ratio during a water diuresis will be discussed later.

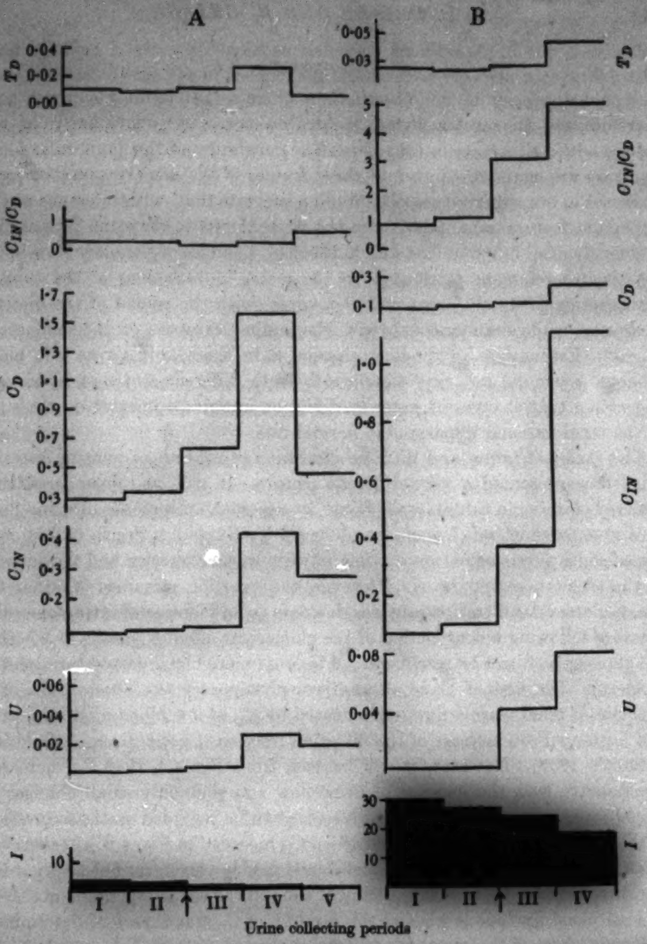

Fig. 4. Changes of inulin clearance, diodone clearance, C_{IN}/C_D ratio and rate of tubular excretion of diodone of normal adult rabbits during the course of a single water diuresis (A) at low blood diodone levels, (B) at high blood diodone levels. Ordinates: I = blood diodone concentration (in terms of mg. I/100 ml. serum), U = urine flow (ml./100 g./min.), C_{IN} = inulin clearance (ml./100 g./min.), C_D = diodone clearance (ml./100 g./min.), T_D = rate of tubular excretion of diodone (mg. I/100 g./min.). Abscissae: urine collecting periods. These periods were not of equal duration. However, their duration not being of significance in the context, they have been represented by arbitrary units. Water given at stage marked by arrow. For discussion of changes observed see text (pp. 456 and 458).

DISCUSSION

It would appear from our observations on rats that inulin clearances, and therefore the glomerular filtration rate, do not vary significantly in this species with an increase in urine flow. The constancy of the glomerular filtration rate was maintained even at the extremes of the urinary excretion rates observed in our experiments. This finding suggests that, with reference to this important feature, renal function in the rat conforms to the same type as that previously established in man and in the dog. Changes of the urine flow in the rat may therefore be attributed, in the main, to variations of the tubular reabsorption of water. This conclusion agrees with the results of the diodone clearances made simultaneously with the inulin clearances on the same series of rats. Estimations of diodone clearances in these animals at low blood diodone levels did not vary significantly with different states of hydration, suggesting that changes of water load had no important bearing on the state of the renal vascular apparatus in normal rats.

The study of inulin and diodone clearance in rabbits at varying rates of urine flow presented a very different picture. It will be remembered that inulin clearances in rabbits were found to rise with an increase of urine flow. This phenomenon, which was also observed by Kaplan & Smith (1935), suggested some physiological association between water excretion and the activity of the glomerular apparatus. However, the question remained whether the rise of glomerular filtration rate was due only to an increase of intraglomerular pressure following a constriction of the glomerular efferent vessels or whether the glomerular filtration rate increased because more blood passed through the glomeruli. The first of these alternatives presupposes the observation of a decrease of renal plasma flow (as indicated by C_D at low blood diodone levels) and a pronounced increase of the filtration fraction (Chasis, Ranges, Goldring & Smith, 1938). However, it will be seen from Fig. 4A that C_D increased significantly with the increase of urine flow and that only small changes of C_{IN}/C_D were observed (C_{IN}/C_D in this case can be regarded as the expression of the filtration fraction). The rise of C_{IN}/C_D recorded in Fig. 4B, i.e. observed in an experiment performed at a high blood diodone level, cannot be regarded as contradictory. C_D in that experiment showed the usual depression observed at high blood diodone levels and the rise of C_{IN}/C_D was therefore determined by the increase of the glomerular filtration rate.

Remaining doubts about the mechanism of the increase of the glomerular filtration rate which occurred with a rise of urine flow may be settled by following the rate of tubular excretion of diodone (T_D) at blood diodone levels high enough to 'saturate' the renal tubules, i.e. at blood diodone levels high enough to ensure the highest possible excretion of diodone by the tubule cells concerned with this process. It will be seen from Fig. 4B that T_D at such high blood diodone levels was found to rise with an increase of the glomerular filtration rate. If the rise of the glomerular filtration rate which occurred with a rise in urine flow had been due to a constriction of the glomerular efferent vessels, the following alternative findings could have been expected: (*a*) a decrease of T_D if the tubular blood supply had been substantially diminished or (*b*) unchanged T_D values if the tubular blood supply had been maintained by the continued circulation of interstitial fluid (Smith, 1943).

The value of T_D obtained at high (saturation) blood diodone levels has been interpreted as referring to the number of 'active' tubules perfused at any moment (Smith, 1943). The rise of T_D at such blood diodone levels, which appeared to accompany an increased glomerular filtration rate, introduces the possibility that the kidney of the normal rabbit responds to an increase of water load with an increase of 'active' nephrons. The increase of renal blood flow at increased water loads, as inferred from the increase of the diodone clearance at low blood diodone levels, would be in accordance with this possibility.

Our results suggest then that, whereas water diuresis in the rat is effected by changes of tubular function, water diuresis in the rabbit is partly due to changes of glomerular haemodynamics. The occurrence of a rise of the glomerular filtration rate and the renal blood flow with an increase of urine flow in the marine seal (Hiatt & Hiatt, 1942), an animal entirely dependent upon its metabolic water for urine formation, indicates that the peculiar response of the rabbit's kidney to an increased water load may not be an isolated instance in the mammalian series.

It would appear from these findings that for investigations bearing on problems of human renal pathology the rat may now be added to the dog as an adequate experimental animal. The use of rabbits, however, would require consideration of the peculiar mechanism of water diuresis in this species.

SUMMARY

Results obtained on rats. (1) Inulin clearances did not change significantly with variations of urine flow ranging from 0·0035 to 0·1030 ml./100 g./min. The mean inulin clearance (= glomerular filtration rate) amounted to 0·351 ± 0·0027 ml./100 g./min.

(2) The mean of the highest diodone clearances observed (Fig. 2, Class I) which would appear likely to be a measure for the minimal renal plasma flow in the normal adult rat was 2·023 ± 0·2903 ml./100 g./min. Diodone clearances obtained at low blood diodone levels did not increase with an increase of urine flow. The estimated mean Tm_D (tubular excretory mass) amounted to 0·1257 ± 0·00269 mg. I/100 g./min.

Results obtained on rabbits. (1) Inulin clearances increased with an increase of urine flow (confirmation of Kaplan & Smith's (1935) findings).

(2) Increases of urine flow were accompanied by a rise of the diodone clearance and a rise of the rate of the tubular excretion of diodone (T_D) both at low and at high blood diodone levels.

A survey of the inulin and diodone estimations in both species of animals suggests that in the rat, as a result of the stability of the renal blood flow and glomerular filtration rate during a water diuresis, the urinary excretion rate is chiefly dependent on changes of the tubular water reabsorption. On the other hand, the results obtained on rabbits suggest that, in this species, an increased water load leads to an increase of the glomerular blood flow and a rise of the glomerular filtration rate. This glomerular mechanism may be assumed to play a part in the regulation of the renal water excretion of normal rabbits.

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OBSERVATIONS UPON THERMAL BURNS: THE INFLUENCE OF MODERATE TEMPERATURE BURNS UPON A PROTEINASE OF SKIN

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This work arose from attempts to find a burn toxin. Reference to the extensive literature has been made unnecessary by the review of Harkins (1942); it is only necessary to mention the views of Robertson & Boyd (1923), who thought that they had found a toxin present and extractable from burnt skin; this was denied by Underhill & Kapsinow (1931). More recently Wilson, Macgregor & Stewart (1938) stated that a toxic protein could be found in oedema fluid from a rabbit taken within 48 hr. from below the site of the burn. Glenn, Peterson & Drinker (1942) also referred to the presence of a toxin in the lymph and burnt area in dogs. The idea that the toxin may be an enzyme, liberated from the site of the burn, does not seem to have been explored. An enzyme would be a dangerous toxin, if liberated from the skin by the act of burning, because it might induce changes of a far-reaching nature in tissue constituents; alternatively, the unmasked local activity of an enzyme remaining in the skin could set up changes contributing to delayed healing. As part of a programme of work initiated to test this hypothesis, the possibility that a proteinase is liberated from the burn was examined. In Part 1 the conditions for extraction of an enzyme from skin are described, and an account is given of the properties of this enzyme: Part 2 deals with its behaviour when the skin is burnt.

PART 1*

Preparation and properties of a skin proteinase

To be of significance as a burn toxin, this proteinase would have to be active in the physiological range of pH, i.e. pH 7·0–8·0. Tissue proteinases so far examined have a pH activity range much more acid than this, with an optimum of approximately pH 5·0, or even more acid; for the literature, the extensive reviews of Bergmann (1942) and Bergmann & Fruton (1941) can be consulted.

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* Part 1 is adapted from a report to the Burns Sub-Committee, Medical Research Council, submitted in January 1944, and Part 2 from a report submitted in July 1944.

In regard to tissue proteinases, as distinct from those in the digestive tract, Hedin (1904, 1922) claimed that, in addition to the β protease with an acid *p*H optimum, an α protease existed in spleen, which was active about the neutral point. Morse (1917) suggested that the α protease of Hedin might be derived from the leucocytes present in the spleen, but Dernby (1918) explained the activity at a neutral *p*H as due to an action by enzymes splitting peptones. Waldschmidt-Leitz & Deutsch (1927) also denied the existence of α protease, identifying a protease of optimum *p*H 4·0 and a peptide-splitting enzyme of optimum *p*H 8·0. Trypsin-like enzymes have been described in some polymorphonuclear leucocytes (Barnes, 1940).

In skin itself, Sexsmith & Peterson (1918) report autolysis of dried and powdered skin (in presence of toluene); there was activity when 'acid to phenolphthalein'. In presence of casein for 48 hr. they got negative results in frog, cat and pig, and positive in human, dog, rabbit and chicken skin. In young animals there was no evidence of autolysis. Yamasaki (1924) claimed that extracts made in the cold for several days from the skin of cadavers contained autolytic proteolytic enzymes. The results seem doubtful. It can be said that there is as yet in the literature no clear proof of the existence of a proteinase which is active at the neutral point, either in skin or even in other tissues. Working mainly with rat skin, but also with human and other skins, we now produce evidence for the presence of this type of proteinase.

METHOD

Preparation of skin. Two methods have been used. In one, skin (12 g. ± 4 g.) was removed from the abdomen and back of shaved adult rats (120–200 g.) immediately after killing (usually with chloroform); after cutting off adherent muscle, sections of 50 μ were made from small pieces in the frozen condition upon a freezing microtome. The thin slices so prepared were transferred to two centrifuge tubes (50 c.c.) and well mixed with ice-cold acetone, approximately 40 c.c. per tube. After standing for 30 min. the acetone was poured off and more added, allowing 30 min. for extraction. Latterly, a third acetone treatment has been used. During the dehydration the slices were kept at 1° C. After removal of the acetone the skin was dried by attachment of the tube to a good vacuum pump for about 15 min.; it was then dried further in a vacuum desiccator, and, when stored in this, showed no loss in activity for 4 weeks. These preparations are called 'acetone-dried skin'. Later it was found that the skin could be obtained in a suitable form by cooling to 2° C. and then cutting thoroughly with sharp scissors until a fine pulp was obtained. This was then dried in cold acetone, as with the microtome sections.

Dry weights (acetone-dried) of 0·3264 and 0·3305 g. (average 0·3284 g.) were obtained from 1 g. original slices so dried.

Earlier experiments were done with the fresh slices taken after cutting with the freezing microtome. These will be called 'fresh skin'.

Estimation of proteolytic activity. At first Folin's method, as used by Anson (1937) for measuring the activity of spleen cathepsin, was tried. This was abandoned mainly because it showed no difference between incubated and fresh samples of skin. It was found that during incubation (both in presence and absence of trichloroacetic acid) a substance estimated by the reagent accumulated. Though not identified, this was probably creatinine arising from creatine.

In all the later work therefore we used van Slyke amino-N estimations for judging the increase in $-NH_2$ groups due to protease action. The technique followed in detail that described by van

Slyke & Peters (1932); the reaction time was from 3 to 6 min., depending upon the temperature. No difference was found if the reaction was extended to 15 min. All estimations were in duplicate. The difference for estimations of amino-N upon the same solutions for amounts of 100–300 μg. was not greater than ±4 μg. Larger differences arise in sampling the skin, but estimations did not vary by more than 10%. Typical values for fresh skin are shown in Table 1; similar differences are found with 'acetone skin'.

In most experiments casein has been used as substrate. It was prepared as follows: 0·5 g. casein (LW., Harrington's) was stirred into 10 c.c. distilled water, 2 c.c. 0·5 *N* NaOH was then added and the whole allowed to stand for 30 min.: 0·5 *N* HCl was then added, to bring to *p*H 6·9±0·1, and the volume made up to 50 c.c. with water. Each experimental tube has contained 0·3 g. 'acetone skin' (or 1 g. fresh skin), together with 5 c.c. casein substrate and 5 c.c. *M*/20 phosphate buffer. At the end of the incubation, or immediately, in the controls, 10 c.c. 0·3 *N* trichloroacetic acid were added, to stop the digestion and to precipitate unchanged protein. After filtration, 5 c.c. were used for the estimation.

Controls giving similar results were done later with the same casein, purified by reprecipitation, and also a few experiments with pseudo-globulin, serum albumen and myogen. The results are expressed as mg. amino-N per 1 g. fresh skin or 0·3 g. 'acetone skin'.

RESULTS

Fresh skin and acetone-dried skin compared. Upon incubation alone, fresh skin slices showed an increase in non-protein amino-N; this was augmented by the presence of casein. Table 1 illustrates these points. The results with fresh skin are apt to be somewhat variable, probably owing to difficulty of penetration of casein to the active centres. Exps. 1 and 2 (Table 1) show the increase in non-protein amino-N which may be expected with the skin alone; Exp. 3 an intermediate value with 0·5% casein. Other values for 3 hr. periods have ranged from 0·84 to 1·44 mg. amino-N per 1 g. There was always an increase when casein was added.

TABLE 1. Proteolytic activity of fresh rat skin incubated with or without casein

Results expressed as increase in amino-N (non-precipitable by trichloroacetic acid) per 1 g. fresh skin. *p*H 6·9±0·1. Varying periods of time and varying concentrations of casein.

				Amino-N (mg.)	
Exp.	Skin	Casein	Incubation hr.	Initial value	Increase over initial
1	Fresh	None	3	0·545	(1) 0·428
					(2) 0·365
2	Fresh	None	3	0·556	(1) 0·776
					(2) 0·706
3	Fresh	0·5%	½	0·580	0·168
			1		0·332
			1½		0·624
			2		0·745
			3		1·073
4	Fresh	0·05%	1	0·636	0·200
		0·2%			0·252
		0·5%			0·444
		1·0%			0·363
5	Fresh (1 g.)	0·5%	1	0·464	0·496
	Acetone dried (0·328 g.)	0·5%	1	0·400	0·392

Exp. 4, Table 1, is one of four similar experiments, which indicated that 0·5% casein is adequate. This concentration was therefore used as a routine in subsequent work.

Exp. 5 indicated that acetone-dried skin had an activity not far removed from that of fresh skin, and this was thoroughly confirmed in subsequent experiments. Further experiments were therefore done with this material, since it could be more easily stored.

Preparation of proteinase in soluble form and effects of salts upon extraction. The most satisfactory methods of preparing the skin for extraction of the proteinase are by cutting sections or mincing finely with scissors. Extracting from acetone-dried unsectioned skin gives unsatisfactory yields, as also from skin frozen in CO_2-alcohol mixture or liquid air. Extracting either fresh or acetone-dried skin in phosphate buffer *p*H 7 or 8, or distilled water, for 45 min. at 38° C., yields no activity in the centrifugate. If, however, the extraction is carried out in a buffered solution (*p*H 7) containing 5% KCl, approximately 50% of the original proteolytic activity of the skin is present in the centrifugate, and extraction is almost complete in 15 min. No more enzyme comes out on re-extraction of the skin. Extraction with 1% KCl is much less efficient. These points are illustrated in Table 2.

TABLE 2. Conditions for extraction of proteinase from skin

F = fresh skin. A.D. = acetone-dried skin. Extraction fluid, *M*/20 phosphate buffer. *p*H 6·9 ± 0·1 with various KCl concentrations and times of extraction. Temp. 38° C. Subsequent digestion with 2·5% KCl for 1 hr., unless otherwise stated. Results expressed as change in amino-N (non-precipitable by trichloroacetic acid) per 1 g. fresh skin or 0·3 g. acetone-dried skin. 5% KCl = 0·670 M.

Exp.	Condition of skin	Skin or extract from	KCl conc. for extraction %	Time min.	Amino-N (mg.) Initial	Amino-N (mg.) Increase over initial
1	F	Skin (before* extraction)	Nil	45	0·642	0·606
		Extract (phosphate alone)	Nil	45	0·396	0·011
2	F	Extract	5	30	0·900	0·688
	A.D.	Extract	5	30	1·044	0·548
3	A.D.	First extract†	5	45	0·520	0·308
		Second extract	2	45	0·160	None
		Skin (after 2nd extraction)	2	45	0·028	0·286
4	A.D.	Skin (after extraction)	5	45	0·072	0·324
		Extract from same	5	45	0·784	0·392
5	A.D.	Extract	5	45	0·404	0·580
	A.D.	Skin after extraction, *p*H 8	5	45	0·072	0·280
		Extract, *p*H 8	5	45	0·652	0·300
6‡	A.D.	Extract	5	15	0·624	0·628
			5	30	0·812	0·680
			5	45	0·872	0·688
7‡	A.D.	Extract	1	30	0·700	0·112
8‡	A.D.	Extract	Nil	30	0·644	0·048

* Digestion without KCl. † Digestion with 1% KCl.
‡ Exps. 6, 7 and 8. Digestion for 1½ hr.

Temperature for extraction. Extraction at 18° C. is only slightly slower than at 38° C. The majority of the experiments were completed at 38° C. before this fact was known.

As a result of these experiments and unless otherwise stated, extracts have been made by incubating mixtures at 38° C. in the proportion of 0·3 g. 'acetone-dried' skin to 5 c.c. buffer solution, $M/20$ phosphate + 5% KCl for 30 min.; 5 c.c. of this solution, after centrifuging, is added to 5 c.c. casein solution, and the digestion then carried out for the requisite period; 10 c.c. 0·3 N trichloroacetic acid is then added, and, after filtering, 5 c.c. of the filtrate is taken for analysis.

The skin extracts keep at 0° C. ± 2·0 for 24 hr. without loss of activity. They have on three occasions been treated with cold acetone: the powder so produced (containing, of course, salts) did not redissolve in water, but still showed some activity. It is hoped to investigate this further, but it is clear that the enzyme is reasonably stable.

Salt concentration for activity. In order to find the optimum salt concentration of the enzyme activity, as distinct from the optimum for extraction, we used dialysed extracts, adding various concentrations of KCl. 2·5% KCl is the optimum for digestion with casein. The dialysed extracts were prepared from extracts dialysed for 4–5 hr. against distilled water in a shaking dialyser, adapted from the Heidelberg Laboratory; this time was sufficient to remove all Cl. In the earlier work, acetone-dried skin was incubated with casein substrate plus NaCl or KCl. There was indication from these experiments that KCl is more effective than NaCl, though active extracts are obtained with the latter.

Activity and pH. For 'fresh' skin, there was little difference between pH 6 and pH 8, probably because the buffer solution fails to penetrate. With the extracted enzyme, the activity at pH 6 was lower (see Fig. 1).

Relation of activity to —SH *groups.* That no —SH group was necessary for activity in the enzyme was shown by a series of experiments, following Hopkins & Morgan (1938), with glutathione (reduced form) and cystine-ester hydrochloride. 4 c.c. of a glutathione solution (1 mg./c.c.) were added to 10 c.c. extracted enzyme and incubated at 38° C. for 30 min. 7 c.c. were then taken for initial estimation and 7 c.c. for incubation with casein. A similar experiment was done with 0·1% cystine-ester hydrochloride. Both experiments were tried aerobically and anaerobically.

In the first two experiments, slight increases were observed in both cases, but, on our taking the precaution of washing the trichloroacetic acid precipitate and adding these washings to the filtrate, the small increases were no longer observed.

Effect of some inhibitors. As might be expected, neither NaF (1 mg./c.c.) nor iodoacetic acid (0·1 mg./c.c.) inhibited the enzyme appreciably.

Specificity. The effects obtained with casein are not diminished after purifying the substrate by precipitating twice at the isoelectric point, a procedure which reduces the amino-N non-precipitable by trichloroacetic acid

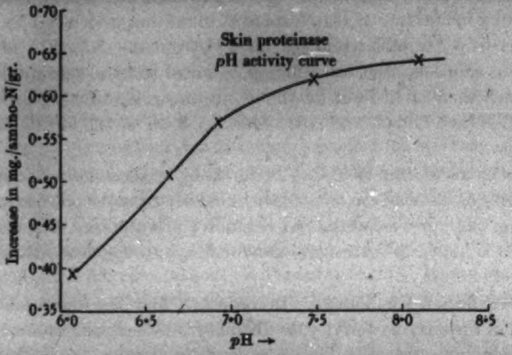


Fig. 1. The *p*H activity curve was determined as follows: extracts from acetone-dried skin were made and were treated with appropriate amounts of acid or alkali (0·5 *N*) to produce the required *p*H; this mixture was then added to similarly treated casein and the final *p*H checked with the glass electrode to ±0·02 *p*H at 20° C.

TABLE 3. Effects upon other proteins

Skin extracts prepared from acetone-dried skin. Results expressed as increase in amino-N (non-precipitable by trichloroacetic acid) per 5 c.c. extract.

		Amino-N (mg.)		
Exp.	Substrate	Incubation time (hr.)	Initial value	Increase over initial
1	None	1	0·892	0·024
	1% serum globulin	—	0·916	0·124
2	None	2	0·892	0·116
	0·5% casein	—	0·904	0·348
	2% albumin	—	0·900	0·304
	0·5% myogen	—	0·896	0·208

TABLE 4. Comparison of dialysed and non-dialysed extracts with and without casein addition. (The dialysed extract was prepared as described on p. 465)

Rat skin incubated for 1 hr. at 38° C. Casein 0·5%. Results expressed as increase in amino-N (non-precipitable by trichloroacetic acid) per 5 c.c. extract.

			Amino-N (mg.)		
Exp.		Casein	Initial	Increase over initial	
1	Non-dialysed	+	0·804	0·412	
	Dialysed	+	(*a*) 0·104	0·468	0·428
		+	(*b*) 0·104	0·388	
2	Non-dialysed	None	0·712	0·152	
		+	0·744	0·488	
	Dialysed	None	0·072	0·140	
		+	0·084	0·468	

to less than 0·02%. Hence the effects upon casein are not due to the action of the enzyme upon any associated polypeptides. Table 3 shows that the skin enzyme also digests other proteins, and is therefore definitely proteolytic.

That the enzyme here concerned liberates amino-N from proteins is proved when the increases in amino-N are compared before and after dialysis. Though dialysis reduced the residual non-protein N values to 10% of that for the undialysed extracts, the increases due to casein addition are practically unchanged, and are therefore due to protein cleavage.

We are aware that the initial changes produced by the proteinase may be followed by some degree of digestion by peptidases present, but this is likely to be small owing to the low concentration of peptides; our experiments showed that the activity of the peptidase was low even in the presence of 0·5 mg./c.c. leucyl-glycyl-glycine substrate. A further argument on this point is that in estimating, over a period of 3 hr., the increase in amino-N, produced by incubation of the skin extract with casein, the curve shows no acceleration in this process, as would be expected if peptidases came into action at some stage during this period. If anything, there is a tendency for the enzymatic action, as judged by amino-N increase, to fall off between the second and third hours; it is possible that this may be due to slight decomposition of glutamine (Chibnall & Westall, 1932); but any such effect appeared to be slight and practically non-existent in the first and second hour, the periods of digestion used here.

Since this proteinase splits a —CONH-linkage, it attacks a peptide group in the protein molecule. Though it has the pH activity curve of trypsin, the enzyme is not trypsin because it does not split Bergmann's specific substrate for this, benzoyl-arginineamide. It is also clearly distinct from peptidases splitting leucyl-glycyl-glycine. This can be proved by differential extraction of the skin.

The activity of extracts on dl-*leucyl-glycyl-glycine and casein.* Two extracts of acetone-dried rat skin were made: first, (*a*) with *M*/10 Ringer phosphate buffer, then (*b*) a re-extraction of the residue from (*a*) with a 5% KCl buffer solution.

The activity of these two extracts was compared on *dl*-leucyl-glycyl-glycine and casein respectively. We found the greater part of the peptide-splitting activity in (*a*) and that of casein in (*b*). The experiment given below illustrates this point. (This is one of three similar experiments made.)

Experiment. (Composition of Ringer phosphate as follows: NaCl 9 g., KCl 0·250 g., $NaHCO_3$ 0·150 g., KH_2PO_4 17·3 g. Mixture is made up to 1254·2 c.c. and approximately 19 c.c. of 20% NaOH added, bringing pH to 7·3.)

Substrate concentration: leucyl-glycyl-glycine (l-g-g) 0·4%, casein 0·5%.

Mixture incubated for 2 hr. at 38° C.

Results expressed as increase in amino-N (non-precipitable by trichloroacetic acid) per 3 c.c. extract.

Extracting fluid	Substrate	Initial	Increase
(*a*) Ringer phosphate	Casein	(*a*) 0·264	0·048
(*b*) Phosphate buffer + 5% KCl		(*b*) 0·136	0·180
(*a*) Ringer phosphate	l-g-g	(*a*) 1·668	0·212
(*b*) Phosphate buffer + 5% KCl		(*b*) 1·564	0·088

Activity of skin proteinase of man and other animals. Similar enzymic activity has been found also in preparations from human, rabbit and guinea-pig skin incubated with casein, so that the proteolytic activity is general for skin; only the guinea-pig has shown variable results (Table 5).

TABLE 5. Skin proteinase of man and other animals

Substrate, casein 0·5% 1 hr. incubation. pH 6·9±0·1. Results expressed as increase in amino-N (non-precipitable by trichloroacetic acid) per 1 g. fresh skin or 0·3 g. acetone-dried skin.

			Amino-N (mg.)	
Exp.	Animal	Skin	Initial value	Increase over initial
1	Man	Fresh	1·240	0·412
2		Acetone-dried	0·216	0·428
3	Rabbit	Fresh	0·448	0·148
		Acetone-dried	0·676	0·264
4		Fresh	0·592	0·148
5	Guinea-pig	Fresh	0·792	0·228
		Acetone-dried	0·716	0·128
6		Fresh	1·172	0·372

In three experiments with human skin (one with dermatome slices and two others with skin from operation), extracts of the same order of activity as those from rat skin were found, though optimal conditions for extraction seem to be different: 5% KCl did not extract from the fresh skin in one case. In experiments on the skin of two rabbits, active preparations were obtained, though they were lower in activity than with rat skin. In some earlier work with guinea-pigs, the skin preparations were inactive or had only slight activity and there was a distinct tendency for instability upon acetone drying; the reasons for this are not yet clear and are being further investigated. In the last four experiments with the guinea-pig, active preparations were obtained.

Effect of temperature upon stability. It was an object of this research to discover whether 'burning' skin at the temperature employed would leave the proteinase intact; the thermal stability has therefore been investigated as follows:

Separate amounts of skin or of preparations equivalent to 1 g. fresh skin in 5 c.c. phosphate buffer were placed in two test-tubes and heated for 5 min. in a water-bath at the required temperature. Heating to this temperature took approximately 1 min. At the end of this period, the tubes were cooled in ice-water; one was treated with 5 c.c. casein solution and incubated for 1 hr. at 38° C., digestion being stopped with 10 c.c. 0·3 N trichloroacetic acid; the second tube was treated immediately with 1% casein solution and trichloroacetic acid as a control.

The results (Table 6) showed that with fresh skin, little inactivation occurred in 5 min. at 60° C. (heating above this temperature was impossible owing to the behaviour of the collagen). With dried skin or extracts from this, serious inactivation did not occur until 70–75° C.; about 25% inactivation took place at 70° C., and about 75% at 75° C.

Immersion of a test-tube containing the extract in boiling water for 5 min. completely inactivated the enzyme.

Table 6. Thermal stability of skin proteinase

Incubation 1 hr. 38° C. Substrate, 0·5% casein. *M*/30 phosphate buffer. Acetone-dried skin and extract from this. Results expressed as increase in amino-N (non-precipitable by trichloroacetic acid) per 0·3 g. acetone skin or 5 c.c. extract.

			Amino-N (mg.)		
Exp.	Skin	Temp. °C.	Initial value	Increase over initial	% change
1	Acetone-dried	Room	0·492	0·372	—
		55	—	0·324	-13
		60	—	0·304	-18
2	Acetone-dried	(*a*) Room	0·524	0·348	—
		70	—	0·218	-37
		(*b*) Room	0·524	0·320	—
		70	—	0·232	-27
		75	—	0·096	70
3	Extract	Room	0·652	0·304	—
		60	—	0·304	0
		70	—	0·240	-21
		75	—	0·060	-80

DISCUSSION

We believe that this is the first convincing demonstration in a tissue of an enzyme with a trypsin-like *p*H activity curve. The enzyme is not trypsin itself, nor, so far as we know, is the activity of the skin due to any enzyme present in traces of plasma left in the skin after death. The following points are against this: the amount of plasma in normal dermis and epidermis is very small; the activity found is greater than for an equal weight of plasma; the enzyme is difficult to extract without cutting the skin into fine sections or into a pulp before acetone drying; in one case, in the rat, extracts similarly prepared from spleen, where blood was present, gave no proteinase activity. For these reasons, we think the enzyme to be a genuine tissue enzyme. By similar methods of extraction we have not found a proteinase active at this *p*H in brain; but we have found it present in kidney, liver and muscle of the rat.

In regard to its detailed action, it is the modern view from the work of Bergmann from 1937 onwards (Bergmann & Fruton, 1941; Bergmann, 1942) that a proteinase is a peptidase of special type; this is based convincingly upon the proof that pepsin will split carbobenzoxy-glutamyl-*l*-tyrosine, trypsin benzoylargininineamide and chymotrypsin peptidases of type R_1-tyrosyl glycine-R_2. In their recent classification they distinguish two types of peptidases, termed endo- and exo-peptidases: these terms are used in the chemical and not the physiological sense to indicate whether the attack is on the internal or external peptide linkage. Our enzyme is presumably an endopeptidase.

PART 2

THE EFFECT OF BURNS ON THE BEHAVIOUR OF PROTEINASE IN RAT SKIN

In Part 1 evidence is produced for the existence of a proteinase in skin, which, though not trypsin, has a similar range of *p*H activity. One feature of the enzyme is its comparative stability to heat. This makes it peculiarly valuable for a study of the problem whether, after burning, such an enzyme can leave the tissue for the general circulation or remain active locally. Before considering this we must be clear what temperatures are involved in a skin burn. Leach, Peters & Rossiter (1943) have delineated two zones in skin which has been burnt, (1) a coagulated zone and (2) a zone damaged by more moderate temperatures. They consider that the latter is the more potentially dangerous so far as enzymes are concerned, if the temperatures producing the pathological effect are not high enough to destroy them. In an analysis of the effect of applied heat upon rat and guinea-pig skin, Leach *et al.* found that 52° C. was a critical temperature for application of heat to the skin surface, and that 55° C. applied for 1 min. would produce scabbing. The actual temperature in the skin below the burning iron is lower than this, and has been shown by Mendelssohn & Rossiter (1944) to lie between 45 and 55° C., making 50° C. the most likely temperature for damage with these short exposures. Since the skin proteinase was not more than 25% inactivated by an exposure to 70° C. for 5 min., it is clear that burns produced by the burning iron at temperatures below this will not inactivate this enzyme. Using the 'iron' we have investigated the effect of moderate temperature burns on the skin proteinase and present the proof that these produce a marked decrease in the proteinase content of the skin.

METHODS

White rats (weight 120–200 g.) were used. After anaesthetizing with ether, the skins were freed from hair with clippers and the animals were burned with the burning iron described (Leach *et al.* 1943), using periods of 1 min. at 54–55 or 60–65° C. Care was taken to use pressures of the iron which should not appreciably occlude the circulation. Some controls were burned immediately after killing with the anaesthetic. In each animal one side was burned and the other was used as a control. For the estimation, the animal was killed either by decapitation or by ether, the burned patches were dissected out and also, as controls, similar unburned patches from the other side. The patches of skin were then cooled to about 2° C., adherent muscle and fat were cut away and the skin finely chopped with scissors. The skin mince was extracted with 5% KCl in phosphate buffer solution as described in Part 1.

The individual values, given in Table 7, give the percentage total change of the proteolytic activity after burning. Estimations were made of the activity of the extracted enzyme and that remaining in the skin. The sum of these two gives the total activity and this was calculated for burned and normal skin. (The values of the extract as estimated were corrected by a factor of 1·6, as the estimation was done on 5 c.c. from a total of 8 c.c. of extracting fluid.)

The experiment given in detail at the end of Table 7 is illustrative of the actual values obtained in these experiments. It shows the percentage difference of the proteolytic activity of normal skin and burned skin, the extract and residual skin activity being calculated independently.

The chart in Fig. 2 gives the data for all experiments in which the animal was allowed to survive for at least 20 min. after burning. It does not include the control experiments in which the animal was killed before burning.

As illustrated in Fig. 2, in the majority of experiments the decrease in enzyme activity for extracts and residual skin were the same (within experimental error). In three experiments, however, there was much less apparent decrease in the extract. This may indicate that the first stage is a liberation of enzyme from the non-extractable state into the surrounding fluid, with a corresponding escape of some enzyme into the lymph.

TABLE 7. Summary of the total changes in proteinase content of rat skin due to burning for 1 min. at 54–66° C.

Percentage change in non-protein amino-N liberated by digestion of casein under standard conditions.

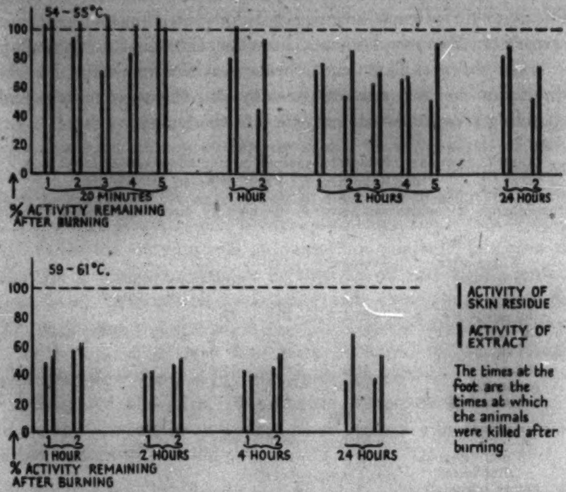
Temp. ° C.	Killed after burning	Average %	Individual values	$S/\sqrt{n}$ S.E. of the mean (ϵ)
54–55	20 min.	− 3·2	+3·5, −1, −14, −8, +3·5	3·42
	60 min.	−23·5	−10·5, −37	
	120 min.	−32·0	−26·5, −31·4, −33·3, −33·3, −35·5	3·4
	24 hr.	−26·1	−14·8, −37·5	
60–61	60 min.	−41·3	−47·5, −35·2	8·0 for mean 50·1
	120 min.	−54·5	−58·5, −50·4	
	240 min.	−54·5	−59, −50	
65–67	9 min.			
	20 min.	−32·1	−30·5, −33·8	
	3 min. (immed. after burns)	− 3	−3	
	Before burning (control)	− 4	−5, −7, +1, −10, +1	4·38

Exp. 1. Illustrative protocol of an individual experiment (selected from those summarized in Table 7), showing the effect of burning upon the enzyme in the KCl extract and in the residual skin solid separately.

Results expressed as increase in amino-N (non-precipitable by trichloroacetic acid) during digestion with casein for 1½ hr., for 0·8 g. residual skin and 8 c.c. extract.

Skin or extract		Temp. of burn ° C.	Killed after burning min.	Amino-N (mg.) Initial	Amino-N (mg.) Increase over initial	Amino-N (mg.) % change
(*a*) Skin:	(i) Control	60–61	120	0·200	0·804	−53
	(ii) Burned			0·100	0·376	
(*b*) Extract:	(i) Control			0·408	0·512	−47·5
	(ii) Burned			0·284	0·208	

Animals burned after death, even at the highest temperature (66° C.) here used, show comparatively small differences in proteolytic activity of the skin even in spite of the heat fixation which was shown by Leach *et al.* (1943) to appear between 55 and 60° C. At 65° C. there is a rapid disappearance of proteinase. At 60° C. the changes after 1 hr. are of the order of 33% and after 2 hr. 53%. Even at 54–55° C., quite marked decreases are present after 2 hr.; there should be no heat fixation here. It is to be noted that there is little change in 20 min. at the lower temperature. In most of the experiments the oedema was between 4 and 14% of the skin weight. There was no oedema present 20 min. or 1 hr. after burning at 55° C.



The times at the foot are the times at which the animals were killed after burning

Fig. 2. Chart showing changes in proteinase activity due to burning at different temperatures for rat skin taken at varying periods after the burn. Each number represents a different experiment; the percentage activity remaining is given for rat skin residue and extract separately.

The haemorrhage observed in the skin was slight after burns of 55° C., although there was some increase 24 hr. after the burn. The haemorrhage was more marked at all periods after burning at 60° C., and again reached a maximum in 24 hr.

The experiments show that there is a gradual disappearance of the proteinase after burning at moderate temperatures which is not due to local heat destruction. There are two possible explanations of this: one is that a factor enters the skin during life which is inhibitory to the enzyme: the other that the enzyme has left the skin. In regard to the first, the control values and those for skin burned at 54–55° C. and taken after 20 min. are practically identical; further, there was no indication of an inhibitory substance in an extract of skin taken after 1 hr., a period during which much fluid will have entered the skin. All the indications are therefore that much of the enzyme has left the skin cells for the general circulation. Nevertheless, in view of our subsequent finding that antiproteinase factors are very active in the plasma, we do not consider it safe to be dogmatic upon this point until we have completed a study of the antiproteinases in this connexion.

DISCUSSION

This work shows that at least one active enzyme leaves the skin comparatively early after burning and at a time coincident with the haemoconcentration. It is significant in this connexion that Perlmann, Glenn & Kaufman (1943) have recently observed a change from the normal electrophoretic pattern in lymph coming from the burned limbs of calves; an additional boundary appeared, which migrated with half the speed of the globulin. From unpublished work in this laboratory by Drs P. Olaffson, Manifold, Rossiter and ourselves, we know that several other enzymes survive exposure to the temperatures causing burns, and could therefore behave like the proteinase reported here. We cannot yet say whether any or all of these enzymes actually cause systemic disturbance and care must be taken not to read into the data more than has actually been proved. But the fact that there is this disappearance must be taken into account in the evidence upon the 'burn toxin' problem. It is also relevant to the problem of metabolic losses of protein after wounds (Cuthbertson, 1942) and after burns (Clark, Peters & Rossiter, 1943), to the problem of shock and to the liberation of Lewis's H substances (1927). The literature upon the action of trypsin and pathological effects, includes work by Roche e Silva (1939) who found that injections of trypsin lower blood pressure in cats, rabbits and dogs and raise tonus in smooth muscle. Blood pressure changes have been described in burns (Harkins, 1942); histamine-like effects (Barsoum & Gaddum, 1936; Rosenthal, 1937) may also be present; it is difficult to disentangle these signs from the phase of haemoconcentration. Grob (1943) claims that subcutaneously injected trypsin will cause skin ulcers in rabbits.

Recently the hypothesis was advanced (Beloff & Peters, 1944) that liberation of the proteinase in intercellular layers of the skin is a factor concerned in the loosening of the epidermis preparatory to blister formation. We suppose that this occurs where Leach *et al.* (1943) found the incipient blister formation. The hypothesis was based partly upon the evidence here presented; but also substantially upon the finding of Medawar (1941) that digestion with trypsin will separate the dermis from the epidermis in sections from human skin made by the dermatome. From the histological evidence, the separation of the skin layers takes place very rapidly, and it may be held that the finding of little change in 20 min. after burning at the lowest temperature here tried is evidence against our hypothesis; but the experiments, which show a marked difference in distribution between the solid and extractable enzyme, are of importance in this connexion. If the skin is taken at an early enough stage, there is not likely to be any difference in the total enzyme content, though the enzyme by then may have passed from inside the cells in to the oedema fluid surrounding them. Liberation of this enzyme could explain the appearance of leucotaxin (Menkin, 1936, 1940; Duthie & Chain, 1939) which is believed to be a polypeptide.

SUMMARY

Part 1

The presence of a proteolytic enzyme has been demonstrated in human, rat, guinea-pig and rabbit skin. The properties of this enzyme in rat skin are as follows:

1. It is active at neutral and alkaline *p*H, the optimum activity being at *p*H 7·5+0·1 and it is not trypsin.
2. It digests casein, serum globulin, serum albumin, myogen and some protein present in the skin itself.
3. Approximately 50% is soluble in 5% KCl solution; the remainder still remains with the solid phase.
4. Maximum activity occurs in the presence of 2·5% KCl.
5. The enzyme is stable towards acetone-drying of skin and, in the extracted form, it is stable towards dialysis against distilled water at 2° C. for 5 hr.
6. It is not inhibited by NaF or iodoacetic acid, nor activated by glutathione (reduced) or cystine (ester).
7. It retains approximately 70% of its activity after heating at 70° C. for 5 min.
8. The proteinase activity here studied can be distinguished from that splitting a tripeptide by differential extraction.

Part 2

1. The behaviour of the 'skin proteinase' has been followed after burns at temperatures varying from 54 to 65° C. for 1 min.
2. Though these temperatures produce little change in the activity of the enzyme through heat inactivation, there is a decrease in the proteinase content of the skin due to burning. At 60° C. this reaches an average of 54% in 2 hr. and at 54° C., 33%. It is suggested that the enzyme leaves the skin.
3. The hypothesis is advanced that the escape of proteinase from the cells contributes to the formation of the vesicle.
4. The relation of the proteinase to 'burn toxin theories' is discussed.

ADDENDUM

Since completion of the above work we have had the opportunity of testing, through the generosity of Dr Bergmann, the effect of the skin proteinase on (*a*) the chymotrypsin substrate, carbobenzoxy-*l*-tyrosyl-glycine amide, and (*b*) *l*-leucyl-glycyl-glycine. (*a*) was not split, (*b*) behaves to the enzyme in a similar way to *dl*-leucyl-glycyl-glycine; hence failure to split this is not due to the presence of the *d*-compound. We therefore conclude that the enzyme is neither trypsin nor chymotrypsin. It is therefore reasonable to think that it is a proteinase which has not previously been described.

Our thanks are due to the Medical Research Council for a grant to one of us (A. B.); and, for part expenses, to the Medical Research Council and also to the Nuffield Committee. We are also grateful for advice to Dr Rossiter and colleagues in the laboratory; to Prof. H. J. Seddon and the Radcliffe Infirmary for human skin; to Mr O'Brien and Mr Leach for important help; to Miss Jenkins for technical assistance; to Dr Stocken for cystine ester hydrochloride and for benzoyl-arginine-amide, and to Dr E. Holiday for specimens of serum globulin, serum albumin and myogen; also to Prof. Chibnall for kind advice.

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THE ACTIVITY OF VAGAL STRETCH ENDINGS DURING CONGESTION IN PERFUSED LUNGS

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In spite of considerable recent work, the factors responsible for cardiac dyspnoea are still far from clear. Christie (1938) has summarized the evidence showing that pulmonary congestion invariably accompanies cardiac dyspnoea, and that afferent vagal impulses from the lungs play a large part in the production of this dyspnoea. His hypothesis is that the vagal 'stretch' afferents record tension in the lung, and that the sensitivity of the endings is increased, as the lungs are more rigid in pulmonary congestion. The alternative view that another set of endings, capable of causing inspiration, is active in dyspnoea, is supported by the work of Partridge (1939) and Hammouda, Samaan & Wilson (1943), and recalls the suggestion of Adrian (1933) that 'deflation' endings may be of importance in pathological conditions.

As the point seemed to have considerable theoretical importance, we have tried to distinguish between these possibilities by recording the activity in vagal single fibre preparations from 'stretch' endings during experimental pulmonary congestion. Considerable difficulties were encountered in consistently producing pulmonary congestion in cats with natural circulation, and we therefore decided to work with perfused lungs in which all the vascular factors were under control.

METHOD

Two cats were used in each experiment, one as a blood donor and the other for the lung perfusion. The first cat was given 1 mg. atropine subcutaneously and was then anaesthetized with ethyl chloride and ether. A tracheal tube was inserted in order to give artificial respiration in the later stages of bleeding. Cannulae were put into the external jugular vein and into the abdominal aorta. 25 c.c. of blood were taken from the aorta and immediately replaced by 25 c.c. of warm Ringer's solution containing 10 μg. adrenaline, which was slowly infused into the external jugular vein. This process was repeated three times (total volume of Ringer's solution infused was 75 c.c.), after which the cat was bled out. The total blood volume thus obtained was about 150 c.c. The blood was carefully defibrinated and filtered five times through muslin.

The second cat was also given 1 mg. atropine and anaesthetized with ethyl chloride and ether. One vagus nerve in the neck (usually the right one) was carefully exposed for a length of 3–4 cm.

A tracheal tube and cannulae in the jugular vein and abdominal aorta were inserted. The defibrinated and filtered blood from the first cat was put into a burette which was connected to the jugular vein. Again, 25 c.c. blood was removed from the aorta and then replaced by 25 c.c. defibrinated blood together with 10μg. adrenaline slowly infused into the jugular vein. This procedure was repeated until the whole of the defibrinated blood had passed through the cat, which was then bled out completely. Artificial respiration was started as soon as the cat stopped breathing and was continued with a small pump stroke throughout the subsequent manipulations. All the blood was once more carefully defibrinated and filtered five times.

The cat's chest was opened in the midline, the pericardium was split, a cannula was inserted into the pulmonary artery (Fig. 1, *PA*), a string was tied firmly around the bases of the ventricles and a second cannula was put into the left auricle (*LA*). The chest wall, which during the preparation had been pulled open, was now allowed to come back almost to its original position leaving a space for the two cannulae. At this point the frame of the moist chamber (described below) was put into position.

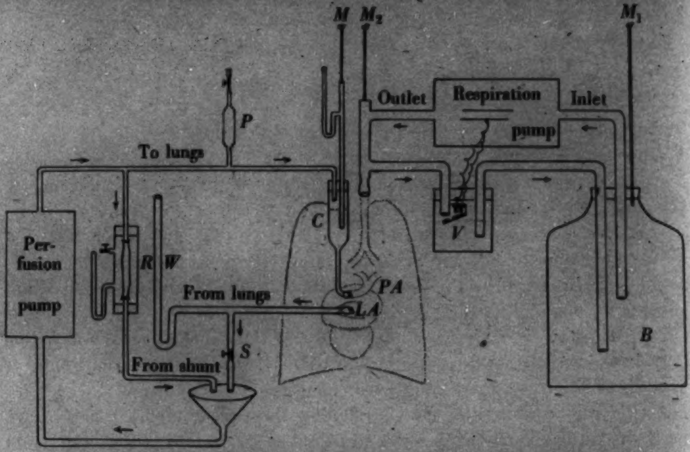

Fig. 1. Diagram of lung perfusion and respiratory circuit. For details see text.

The lungs were perfused with a Dale-Schuster pump. The tube leading to the arterial cannula was connected by a side tube to a 20 c.c. pipette (*P*) the other end of which was closed and which was filled with air, thus acting as an air cushion. A second side tube led to a shunt through an artificial resistance set at 50 mm. Hg. Another air cushion was provided in the arterial cannula (*C*) itself: the glass tube inserted in the pulmonary artery was bent and widened into a tube of 2 cm. diameter held vertically. This cylinder was partly filled with blood, partly with air, and closed with a rubber stopper through which one glass tube let the blood in, while another was connected with a mercury manometer and also with a membrane manometer (*M*) for optical recording of the pulmonary arterial pressure. The venous pressure was measured by a water manometer (*W*) connected with the cannula in the left auricle. The pulmonary arterial pressure could be increased at will by raising the resistance in the shunt and/or by increasing the pump stroke. The venous pressure could also be raised at will by obstructing the outflow from the venous cannula with a screw clamp (*S*). At the beginning of each experiment the venous pressure was zero and the pulmonary arterial pressure 18–20 mm. Hg.

Cats' lungs, when perfused with defibrinated blood, easily become oedematous. Modrakowski (1914) first pointed out the necessity for washing out the blood vessels with defibrinated blood. In our own experiments, not only this precaution but also the reduction in pulse pressure by air cushions, the avoidance of excessive arterial pressure by including a shunt, and finally the inflation of the lungs with a small volume of air secured good results. In large and strong cats the perfusion could be maintained for 4–5 hr. without lung oedema developing. But even with the precautions described we still had occasional failures in which lung oedema began within half an hour of starting the perfusion. We are reporting here the results of six successful perfusions with a total of twelve reversible congestions.

The lungs were inflated with known volumes of air (40–80 c.c.) from a rigid pump similar in principle to the Starling Ideal Pump, running at 14 strokes per min. This allowed about 2 sec. for the completion of expiration. The rotary expiratory valve of the Starling Ideal Pump was replaced by an electromagnetic valve (*V*) worked by a commutator on the shaft of the pump. This valve was absolutely airtight. In order to detect any variation in the amount of air in the lungs at the end of expiration a 10 l. bottle (*B*) was connected to the expiratory outlet and to the pump inlet, thus producing a closed circuit. A sensitive membrane manometer recorded changes in pressure in this bottle and was calibrated by withdrawal of known amounts of air from the circuit. A second membrane manometer connected to the tracheal tube recorded the pressure during inflation and indicated gross changes in the resistance to inflation due to narrowing of bronchioles or changes in the lung parenchyma.

A moist chamber was required for the dissection of the single vagal fibres in the neck. The animal lay on a warm table. A wooden frame had pieces cut out to fit over the cat's head and belly. The right side of the frame had an opening with a rubber curtain through which the operator could pass his hands to dissect the vagus. Through a hole in the left side of the box a tube was passed and connected with the venous cannula. The glass top consisted of two glass plates which were slid on from both sides as soon as the perfusion had been started; they had a hole cut out to fit around the arterial cannula. Any spaces left were plugged with cotton wool. Warm moisture was produced by an immersion heater in a small dish of water covered with wet muslin.

Single fibre preparations were obtained by cutting down the vagus nerve with sharp needles. In spite of the absence of blood supply to the nerve and to the tissues of the neck it was usually possible to obtain responses from a number of pulmonary stretch afferents. Occasionally, when no response could be obtained, conduction was re-established by dissecting away fatty tissue surrounding the nerve in the anterior mediastinum and exposing the whole length of the nerve to air. In one dog, the lungs of which were successfully perfused, very few fibres were found to be alive in the neck and these rapidly failed after dissection. As pointed out by Daly & von Euler (1932), in connexion with the survival of vasomotor fibres to the lungs, a bronchial blood supply is essential in the dog. The fact that the nerve trunk and the tissues in the hilus of the lung are much thicker in the dog than in the cat probably accounts for the difference in survival of the vagal fibres. In these perfused lung preparations we have never found any activity in other than stretch fibres. Depressor fibres are known to have a smaller diameter and to belong to the δ group (O'Leary, Heinbecker & Bishop, 1934); such fibres are very sensitive to oxygen lack (Grundfest, 1939), and are unlikely to be active in these conditions. Nerve impulses were recorded by means of the usual amplifier, cathode ray tube and camera. Artefacts arising from the perfusion apparatus were avoided by earthing all parts which were electrically connected to the cat. The moist chamber was screened as far as possible and earthed.

RESULTS

The discharge from the stretch afferents from perfused lungs was in all respects similar to that seen in cats with normal circulation and in which the lungs were inflated with the same pump (Whitteridge & Bülbring, 1944). The relation between degree of inflation and frequency of vagal impulses was normal. In successful perfusions the frequency of discharge with a constant pump stroke remained steady within ±2 % over 20 min. At the end of some perfusions, when the lungs were not collapsing as well as in the beginning, there was a tendency for the frequency to rise progressively in spite of the constant pump stroke. When this occurred it was found that the pressure of air in the reservoir bottle began to fall, indicating that there was some retention of air in the lungs.

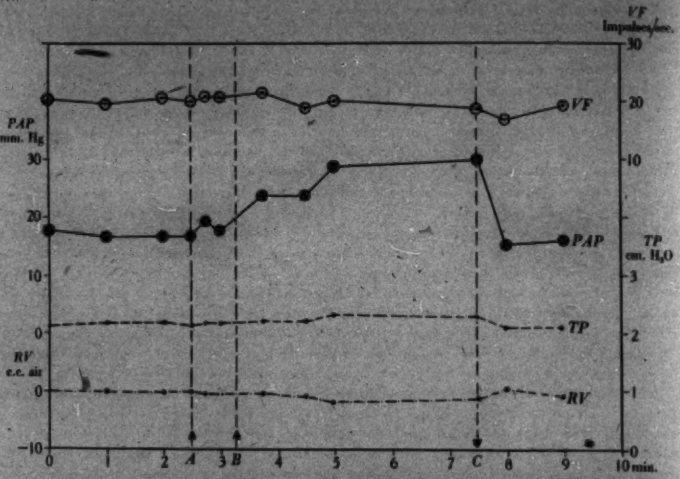

Fig. 2. Lung perfusion. Pulmonary congestion produced by raising the venous pressure to 10 cm. H_2O at *A*, and to 20 cm. H_2O at *B*. Venous obstruction removed at *C*. ◎ (*VF*) = Peak frequency of discharge in a single vagal stretch afferent. ● (*PAP*) = Pulmonary arterial pressure. ------ (*TP*) = Tracheal pressure at full inflation. -·-·-·- (*RV*) = Pressure changes in the reservoir (these indicate changes in the opposite direction in volume of air in the lungs at expiration).

Congestion was produced in three ways: either by impeding the venous outflow up to complete occlusion, or by raising the arterial pressure (maximum 60 mm. Hg), or by impeding the venous outflow simultaneously with raising the perfusion pressure. During congestion the shunt was shut.

The effect of simple venous congestion, corresponding to 'back pressure' from the left atrium, is illustrated in Fig. 2: The change in pulmonary arterial pressure was at first slight owing to the known capacity of the lungs to accommodate large amounts of blood. It took about 3 min. before the pulmonary arterial pressure became steady at its new higher level. The frequency of discharge in the vagal stretch fibre, however, showed no significant change.

At the beginning of a period of congestion there was always an initial increase in the resistance of the lungs to inflation. This was recorded as an increase in the intratracheal pressure at the peak of inflation. In most experiments this increased resistance was maintained throughout the period of congestion and disappeared at the end.

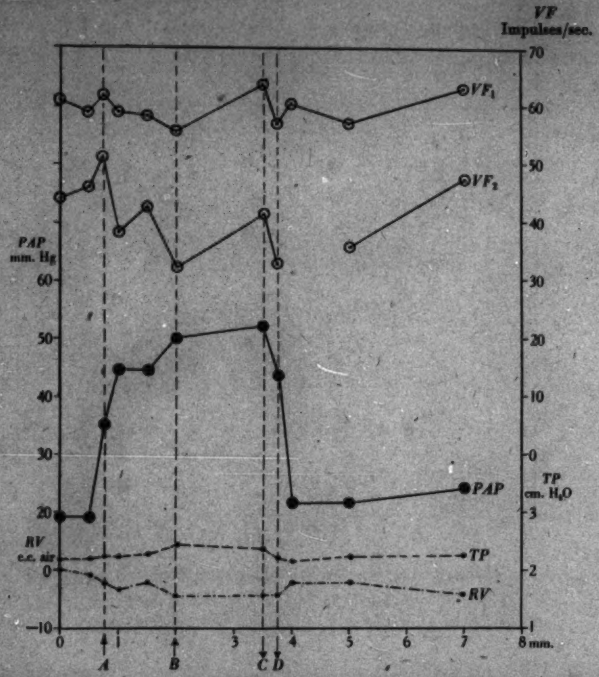

Fig. 3. Lung perfusion. Congestion was produced by raising the pump stroke at *A* and stopping the venous outflow at *B*. At *C* the venous clamp was removed and at *D* the pump stroke was lowered to the original level. Indications as Fig. 2.

During congestion there was also evidence of a retention of up to 4 c.c. air in the lungs, indicated by a decrease in pressure in the large reservoir bottle. At the end of the period of congestion the retained air was expelled.

In three experiments in which congestion was produced by impeding the venous outflow (nos. 1, 2, 8 in Table 1) there was once a small increase, once a small decrease and once no significant change in the frequency of stretch impulses. Three experiments were then carried out in which the perfusion pump stroke was raised (nos. 3, 4, 5 in Table 1), thus increasing the pulmonary arterial pressure only, but not the venous pressure. At a pulmonary arterial pressure of 40 and 60 mm. Hg there occurred some increase in the resistance to inflation, a small retention of air in the lungs and a small increase in frequency of stretch impulses.

Much more severe congestion of the lungs was produced by raising the perfusion pressure and also impeding the venous outflow. Fig. 3 illustrates one such experiment in which the venous outflow was in fact completely occluded.

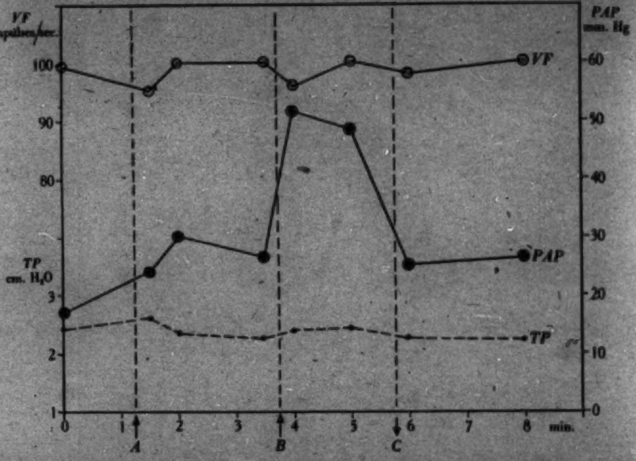

Fig. 4*a*. Comparison of the behaviour of vagal stretch endings (*a*) during congestion, (*b*) during exposure to 3 % trichlorethylene. Fig. 4*a* at *A* venous pressure raised, at *B* pump stroke increased, at *C* return to normal conditions. Fig. 4*b* trichlorethylene administered from *A* to *B*.

There happened to be two fibres the frequency of which could be followed. In spite of an increase of 30 mm. Hg in the pulmonary arterial pressure the frequency of discharge in both fibres showed insignificant changes. Even this maximum congestion did not produce pulmonary oedema and the lungs withstood another period of congestion 1½ hr. later.

Fig. 4*a* shows another experiment in which the venous pressure was raised and the pump stroke was then increased. Though the pulmonary arterial pressure rose from 17 to 51 mm. Hg the frequency of impulses dropped temporarily from 100 to 96 per sec. and never rose above the initial level throughout the period of congestion.

Contrasting sharply with the observations described is an experiment shown in Fig. 4*b* where 3 % trichlorethylene was administered for 3½ min. The pulmonary arterial pressure fell slightly and the resistance to inflation also dropped slightly but the frequency of discharge in the vagal fibre rose from 106 to 166 per sec. The fact that the pulmonary arterial pressure fell, while the output of the perfusion pump remained constant, indicates a vasodilator action of trichlorethylene. Fig. 5 shows three of the actual records from an experiment similar to that in Fig. 4*a*.

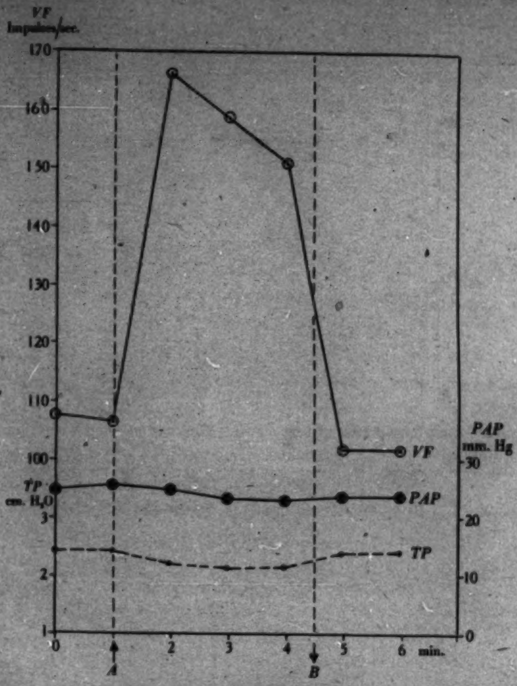

Fig. 4*b*. For explanation see Fig. 4*a*.

In Table 1 the mean of the peak frequencies of discharge from stretch receptors during congestion has been compared with the mean before and after congestion in each experiment. It will be seen that of the fourteen fibres studied there were significant increases in five, a significant decrease in one, while the remaining eight showed no significant change. All the differences were very small and did not exceed 10 impulses/sec., whereas the increase caused by trichlorethylene amounted to 60 impulses/sec. and even larger increases have been seen when anaesthetics were administered to intact animals (Whitteridge & Bülbring, 1944). In all cases, including the experiment

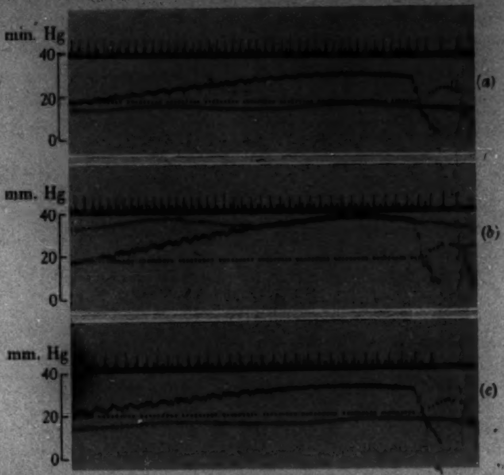

Fig. 5. Records during lung perfusion (*a*) before, (*b*) during, (*c*) after congestion of lung. The second half of inflation and the beginning of expiration is shown in each record. Records in (*a*) from above downwards: (1) impulses in two vagal stretch fibres (retouched); (2) tracheal pressure; (3) time marker $\frac{1}{15}$ and $\frac{1}{150}$ sec. superimposed on record of reservoir volume; (4) pulmonary arterial pressure.

TABLE 1. Peak frequency of impulses in vagal stretch fibres
Significant increase +, significant decrease −, no significant change 0

Exp.	Before and after congestion	During congestion	Difference	Result
1	102·8 ± 0·52 (2)	108·1 ± 0·87 (2)	+5·3	+
2	96·1 ± 0·2 (2)	92·7 ± 0·72 (3)	−3·4	−
3	93·2 ± 0·87 (4)	95·3 ± 1·07 (3)	+2·1	0
4	95·0 ± 0·40 (4)	100·1 ± 0·4 (2)	+5·1	+
5	91·7 ± 0·63 (4)	98·9 ± 0·45 (2)	+7·2	+
6	40·4 ± 1·98 (3)	31·2 ± 4·04 (4)	−9·2	0
7	91·7 ± 7·60 (5)	97·4 ± 0·56 (4)	+5·7	0
8	19·7 ± 0·52 (6)	20·2 ± 0·51 (6)	+0·5	0
9 A	43·5 ± 1·64 (5)	49·7 ± 1·84 (5)	+6·2	+
9 B	59·5 ± 0·70 (6)	62·6 ± 1·32 (5)	+3·1	0
10 A	45·8 ± 1·01 (3)	39·7 ± 2·87 (6)	−6·1	0
10 B	60·2 ± 1·00 (5)	59·3 ± 1·23 (6)	−0·9	0
11	99·2 ± 0·60 (3)	98·3 ± 1·07 (5)	−0·9	0
12	110·4 ± 0·55 (4)	119·1 ± 0·78 (5)	+8·7	+
		Weighted mean difference	+1·66 ± 0·871	

in which there was a significant fall in frequency of discharge, there was a retention of air in the lungs. By altering the stroke of the respiration pump it was found that an increase of 4 c.c. of air would be expected to produce an increase of about 2–7 impulses/sec. in different experiments. The mean differences for all fibres (weighted for the number of observations) was an increase of 1·66 impulses during congestion. From Fisher's Table of *t* (Fisher, 1941), *P* lies between 0·1 and 0·05, i.e. this difference is just below the level of significance and is certainly not more than would be produced by the retention of air. The increased scatter in the majority of cases during congestion suggests that the distribution of air to the different respiratory units has become less regular. It seems safe to assume that in Exp. 2 access of air to the relevant respiratory unit had been hindered, and it follows that other respiratory units must have received more air than before.

Discussion

When this work was started we believed that, in conditions in which pulmonary congestion plays an important part, there probably is an increased sensitivity of vagal stretch endings. We were rather surprised to find that the frequency of impulses in vagal stretch fibres showed increases as small as 7 % in our earlier experiments in which congestion was produced merely by impeding the venous outflow, and we imagined that we were not obtaining a sufficient degree of congestion. With normal capillary permeability one would expect pulmonary oedema to begin when the pressure inside the capillaries is just greater than that of the plasma proteins. In his perfusion experiments Modrakowski (1914) found that the lungs could tolerate pressures up to 80 mm. Hg on the arterial side and 35 mm. Hg on the venous side for short periods without the appearance of pulmonary oedema. In our experiments we approached these limits as closely as we dared and precipitated pulmonary oedema in two instances. Nevertheless we failed to observe more than a trifling change in the frequency of vagal stretch impulses.

The appearance of the paper by Trowell (1943) reminded us of the patchy interference with air inflow which may be produced by bronchial congestion. This he observed to follow very slight degrees of pulmonary congestion. In our experiments, in which the bronchiolar capillaries may have filled from the pulmonary veins, there was certainly some interference with the collapse of the lungs, as 2–4 c.c. of air was usually retained.

It is likely that this interference with airflow results in redistribution of air to the alveoli so that some may collapse and others show some degree of compensatory distension. It follows that a true picture of the effects of any agent causing congestion can only be obtained by observing as large a number of fibres as possible and by measuring the mean change in frequency of discharge. From Table 1 it is clear that there is no evidence for an increased sensitivity of stretch endings during the greatest possible degree of congestion up to the point of lung oedema.

A more prolonged congestion was impracticable as it is impossible to rely on longer survival of a single fibre preparation.

The question may well be asked whether conditions in an open chest with inflation of the lungs with positive pressure are comparable with those in a closed chest. In fact, Drinker, Peabody & Blumgart (1922) found during congestion a considerable increase in the resistance of the lungs to inflation when the pleura remained intact, and a much smaller change with the pleura open. Christie (1938) ascribes the whole of the reflex effects of congestion to these observable changes in resistance to inflation. But in our experiments there was consistently an increase in resistance to inflation at the beginning of congestion, at which time the vagal frequency may rise or fall.

Since the mechanical conditions of our experiments were probably comparable with those in the perfusion of dog's lungs carried out by Daly, Ludany, Todd & Verney (1937), we think it highly unlikely that in their experiments any increase in sensitivity of stretch endings occurred. They found, however, that pulmonary congestion, produced by impeding the venous outflow, not only reflexly affected the systemic blood pressure but also stimulated the separately perfused respiratory centre. Both an increase in respiratory rate and an increased inspiratory tone were seen. We are therefore forced to the conclusion that there must be a second set of afferent fibres in the vagus which is capable of accelerating the respiration. This is the view put forward by Partridge (1939) and by Hammouda *et al.* (1943). So far, the only known method of increasing the sensitivity of stretch endings is by the administration of volatile anaesthetics (Whitteridge & Bülbring, 1944). A re-investigation of the rapid breathing caused by multiple starch embolism (Walsh & Whitteridge, 1945) has shown that there is no consistent sensitization of stretch endings.

SUMMARY

1. A method is described for the perfusion of the cat's lungs and for simultaneously recording the activity in single vagal afferent fibres.

2. The lungs were congested for periods of from 2 to 5 min. by impeding the venous outflow up to complete occlusion or by raising the pulmonary arterial pressure up to 60 mm. Hg. Neither of these two methods, nor the combination of them, caused any significant change in the frequency of discharge from vagal stretch endings.

3. Reasons are given for believing that pulmonary congestion in the intact chest is not accompanied by increased sensitivity of vagal stretch endings, any more than in the perfused lungs.

4. These results cast doubt on a current hypothesis to explain cardiac dyspnoea. Reflexes arising from other than stretch afferents in the vagus may be involved.

The authors wish to thank Mr H. W. Ling for his careful technical help, and the Christopher Welch Fund Trustees for defraying the cost of photographic material.

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5 *February* 1944

Reversible adsorption of haemoglobin at an oil/water interface.

By G. S. Adair, J. J. Elkes,* A. C. Frazer,* J. H. Schulman and H. C. Stewart.* *From the Physiological Laboratory, Cambridge; Pharmacology Department, Birmingham; Colloid Science Department, Cambridge; and the Physiology Department, St Mary's Medical School, London*

We have previously described the adsorption of proteins to charged oil/water interfaces as a *p*H-conditioned phenomenon. Haemoglobin behaves similarly, being adsorbed to a negatively charged emulsion in acid media up to *p*H 6·8, and above this value to positively charged interfaces.

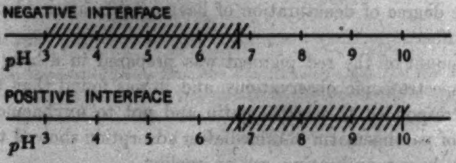

Fig. 1. The adsorption of haemoglobin on oil/water emulsion. The cross-hatching represents adsorption and flocculation.

The point of change-over is in accord with the isoelectric point determined by Adair [1934]. The adsorption of protein on to emulsion results in the formation of floccules which with haemoglobin have a deep red colour.

If the red floccules formed at *p*H 4·6 and washed free of any unattached haemoglobin are transferred to a buffer of *p*H 8·6, the red haemoglobin is immediately released and the emulsion redispersed. If this mixture is centrifuged at 10,000 r.p.m., the oil droplets separate as a white layer leaving a clear red solution below.

The negative interface, used in these experiments, was obtained with olive-oil emulsion stabilized with 0·2% hexadecyl sodium sulphate; the positive interface with a similar emulsion stabilized with 0·2% hexadecyl trimethyl ammonium bromide.

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* Sir Halley Stewart Research Fellow.

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The effect of reversible adsorption on the haemoglobin molecule.

By G. S. Adair, J. J. Elkes,* A. C. Frazer,* J. H. Schulman and H. C. Stewart.* *From the Physiological Laboratory, Cambridge; Pharmacology Department, Birmingham; Colloid Science Department, Cambridge; and the Physiology Department, St Mary's Medical School, London*

The effect of reversible adsorption of haemoglobin to the oil/water interface on its molecular structure was studied by spectroscopy and solubility tests.

Before adsorption. The original haemoglobin solution gave two absorption bands at 578 and 540, and the addition of sodium hydrosulphite gave a purple pigment with one broad absorption band at 565. These spectra correspond with those of oxyhaemoglobin and reduced haemoglobin. The pigments were freely soluble in a 1·5 M phosphate buffer at pH 6·8.

After adsorption. The red pigments gave three very faint absorption bands, one in the red and two in the green part of the spectrum, suggesting either a mixture of oxyhaemoglobin and alkaline haematin, or parahaematin [Keilin, 1926].

The addition of sodium hydrosulphite gave rise to the unmistakable spectrum of haemochromogen with well-marked bands at 558 and 520. These pigments were precipitated by 1·5 M phosphate buffer at pH 6·8. These results indicate some degree of denaturation of haemoglobin after adsorption at an oil/water interface.

A larger amount of the red pigment was prepared in a later experiment, to facilitate spectroscopic observations, and it was found that the bands in the green corresponded to parahaematin and not to oxyhaemoglobin. This soluble form of parahaematin obtained after adsorption showed the reversible colour change, brown on heating, red on cooling.

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The proteinase of skin.

By A. Beloff and R. A. Peters. *The Department of Biochemistry, Oxford*

A search for an enzyme of this nature has been made in connexion with work upon thermal burns in the attempt to settle whether liberated enzymes can act as 'burn toxins'. We have proved that there is present in the skin of man, the rat, rabbit and guinea-pig a genuinely proteolytic enzyme, with an activity pH curve resembling that of trypsin. The enzyme is extracted from finely divided 'acetone-dried' skin with 5% KCl in phosphate buffer solution (pH 7·0); the latter alone does not extract it. Even under the best conditions approximately 50% of the proteolytic activity remains behind. The enzyme is relatively stable to heat, not more than 25% inactivation taking place after 5 min. heating at 70° C.; hence it is not destroyed by a temperature which will produce a severe burn when applied for 1 min. [Leach, Peters & Rossiter, 1943].

Shortly after burning in the rat, the proteinase content of the skin is diminished, both that of the extractable enzyme and of the enzyme still left unextracted; this suggests that the proteinase is liberated after heating. Since Medawar [1941] has shown that trypsin will liberate dermis from epidermis in human skin, it is possible on our facts to advance the hypothesis that the liberated proteinase prepares the skin for blister formation. Table 1 gives some illustrative results.

TABLE 1. Changes in amino-N (by Van Slyke method) in extracts from burned and control patches of skin (rat), due to proteinase action on casein. Burning by application of burning iron for 1 min. Anaesthetic ether. Skin removed after killing, chopped with scissors and extracted.

Exp.	Temp. of burning iron ° C.	Time killed after burning	Condition of skin	Time of casein digestion hr.	Extract from patch	Amino-N* Initial	Amino-N* Increase	% decrease
1	61·2	2 hr.	Slight haemorrhage and oedema (10%)	2	Control	0·444	0·612	56
					Burned	0·272	0·268	
2	65–67	20 min.	Small haemorrhagic patch without oedema	1	Control	0·332	0·268	30
					Burned	0·272	0·188	

* Estimated in comparable amounts of digestion mixture, mg. NH_2N/5 c.c. extract.

This work was done with grants from the Burns Sub-Committee of the War Wounds Committee, Medical Research Council. We are indebted to Miss Jenkins for technical assistance.

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PROCEEDINGS

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18 *March* 1944

Appreciation of the colour of small objects. By H. Hartridge.

St Bartholomew's Medical College

It is well known that difficulties are experienced in comparing the colour or intensity of one small object with that of another. It is not so generally known that the colours of such objects are considerably modified so that the observed colour differs materially from the true colour. The conditions under which these modifications occur may be summarized as follows:

(1) *Errors in colour appreciation due to the chromatic aberration of the eye.* It is found by experiment that white and lemon-yellow objects on a black ground tend to be confused because the violet halo produced by the first is not readily appreciated and therefore causes confusion with the second. It is similarly found that black and purple objects on a white ground are confused because the purple halo produced by the second object is not appreciated and therefore tends to cause confusion with the first.

When small colourless objects are at different distances from the observer they acquire colours which are caused by the chromatic aberration of the eye. Nearer objects are given a blue or blue-green colour, whereas more distant objects are given an orange or yellow colour.

(2) *Errors in colour appreciation produced by the use of common optical instruments.* In the majority of optical instruments the correction of chromatic aberration leaves uncorrected what is called the secondary spectrum. Thus in microscope objectives the colours of the secondary spectrum are usually apple green and purple. But there are microscope objectives in which the colours are blue-green and rose pink. In consequence of the secondary spectrum, objects slightly out of focus are given a spurious colour. Thus the markings on diatoms are frequently highly coloured because of this effect and this is fairly generally known. What is not so universally known is that objects which refract light, for example, droplets of fluid of high refractive index in a medium of lower refractive index (for example xylol in water), acquire a colour which is due to the secondary spectrum of the objective. Thus in the case cited the xylol droplets appear apple green with a narrow purple surround; the latter however is frequently masked by other aberrations present in the objective.

In the case of small coloured objects the same modification of tint can take place, thus red blood corpuscles which are naturally straw yellow or pale orange in colour become modified to greenish yellow or even in rare cases to yellowish green when viewed through an ordinary microscope. Similar alterations of other coloured objects occur.

The form of the retinal image. By H. HARTRIDGE.

St Bartholomew's Medical College

The following facts are in favour of the view that the eye suffers from chromatic aberration: (1) Measurements by Fraunhofer & Wolf [1888], and others show that colour error is not corrected in the eye as it is in many optical instruments. (2) The cobalt blue glass experiment [Tscherning, 1904] demonstrates chromatic aberration to be present. (3) Simultaneous contrast will enhance rather than neutralize the colour error present. (4) The error cannot be lessened to any appreciable extent by scattered light. The following facts are against the view that the eye suffers from chromatic error: (1) When the eye is used in ordinary vision coloured fringes are almost, if not entirely, unobserved. (2) The resolving power of the eye for fine detail compares favourably with that given by a fully corrected lens. (3) The use of a correcting combination of crown and flint glass [Helmholtz, 1909] improves the performances of the eye hardly at all. (4) The employment of monochromatic light (e.g. sodium light [Luckiesh, 1920]) improves the visual acuity of the eye to only a trifling extent.

The explanation of these apparently contradictory results is provided by the fact that two phenomena come into play to reduce the amount of colour error present. One operates at small apertures, namely, the correction of chromatic aberration by diffraction; the other operates at large apertures, namely, the retinal direction [Stiles & Crawford, 1933] effect. With regard to the first it is found that the rays which form the smallest aberration disks on the retina are those which undergo the greatest amount of diffraction and vice versa, the consequence being that the one tends to neutralize the other. The neutralization, however, is not complete for the extremely long red rays or for the extremely short violet ones, thus explaining the cobalt blue glass experiment.

With regard to the Stiles and Crawford phenomenon this operates at pupil diameters larger than about 5 mm. There would seem to be some factor in addition to this which operates particularly at medium apertures. It is not known at the present time what this is. Investigations on this point are, however, in progress.

The relationship of lipolysis to emulsification of triglyceride in the small intestine. By A. C. FRAZER* and H. G. SAMMONS.† *From the Pharmacology Department, Birmingham*

In vitro experiments show that a triple combination of monoglyceride/fatty acid/bile salt provides an emulsifying system which is effective over the whole physiological *p*H range in the small intestine. Bile salts occur in the normal intestinal contents in suitable concentration (less than 1 %) during fat absorption. The monoglyceride and fatty acid can be formed by hydrolysis of triglyceride. The relationship of lipolysis and emulsification is illustrated by two groups of experiments.

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(*a*) In vivo *experiments*. If the finely emulsified olive oil is collected from the small intestine of the rat, the particle can be shown to be negatively charged and resistant to acid at *p*H 4·0. If oleic acid is administered, it is not finely emulsified in the small intestine. Sodium oleate stabilized emulsions of paraffin cream and break if left in the intestine for a few hours.

(*b*) In vitro *experiments*. If olive oil and lipase solution buffered to *p*H 6·5 are incubated at 37° C. lipolysis ensues. If this solution is shaken at hourly intervals, emulsification does not occur. If 0·5% of bile salts is added emulsification occurs spontaneously. Spontaneous emulsification at this *p*H does not occur with fatty acid and bile salt alone but only when they are combined with monoglyceride.

The possible effect of *p*H, time, and the nature of the substrate on monoglyceride formation during lipolysis is being investigated.

The mechanism of emulsification of triglyceride in the small intestine.

By J. J. Elkes,* A. C. Frazer,* J. H. Schulman and H. C. Stewart.* *From the Pharmacology Department, Birmingham; Colloid Science Department, Cambridge; and Physiology Department, St Mary's Hospital Medical School, London*

Triglyceride is finely dispersed in the small intestine and paraffin emulsion of similar dispersion is readily absorbed [Frazer, Stewart & Schulman, 1942]. The mechanism by which triglyceride is finely emulsified in the small intestine has not been satisfactorily explained or demonstrated.

The possible factors in the small intestine which might be concerned in emulsification appear to be fatty acid, monoglyceride, soap, cholesterol, and bile salts. Phospholipin has been excluded since it can be shown by experiment with flocculation reactions and lecithinase that it does not form an essential part of the stabilizing interfacial film in intestinal emulsions [Elkes & Frazer, 1943]. The possible emulsifying agents have been studied singly, and in double and triple combinations. The criteria considered necessary for the assessment of a satisfactory intestinal emulsifying system are:

(i) Effective action over a *p*H range of 6·0–8·5.

(ii) Spontaneous emulsification without violent agitation.

(iii) Particle size of less than 0·5μ diameter.

(iv) Stability for a period of 3 hr.

In all the experiments only one system was found to satisfy these criteria completely. This system is the triple combination of monoglyceride/fatty acid/bile salt. This system produces excellent emulsification over the whole of the physiological *p*H range found in the small intestine. It is suggested that fatty acid supplies the necessary charge on the monoglyceride stabilized particle at the more alkaline end of the *p*H range, while the fatty acid/bile salt complex provides the charge when the ionization of the fatty acid is depressed in more acid media.

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* Sir Halley Stewart Research Fellow.

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The renal tubular reabsorption of phosphate in man.

By J. A. Barclay, H. G. Bray and W. T. Cooke. *The Departments of Physiology and Biochemistry (Birmingham Medical School) and the Birmingham United Hospital*

Some measure of the rate of tubular reabsorption of phosphate would be of value, not only from the viewpoint of renal physiology, but also as a means of assuring the extent of the renal lesion in defects of phosphorus metabolism.

The experiments were carried out on ten students and four hypertensive patients, observations being made over a total of 62 periods. 200–300 c.c. of isotonic phosphate at *p*H 7·4 were administered intravenously. Harrison & Harrison [1941 *a*] point out that phosphate given intravenously enters into organic combination but show that at the end of an hour it is present once more in the inorganic form; the first blood sample was therefore not taken before an hour had elapsed. 45 min. after the phosphate injection Inulin and Diodone were administered in nine of the subjects by the subcutaneous route as a 25% solution of Inulin and a 16% solution of Diodone [Findley & White, 1940], or by continuous intravenous drip at the rate of 4 c.c./min., a total of 400 c.c. of 0·5% Inulin and 0·3% Diodone being given. A 'priming' dose of 1·3 c.c. 35% Diodone and 30 c.c. 10% Inulin was given when the drip was started.

Harrison & Harrison [1941 *a*, *b*] showed that in the dog the rate of reabsorption of phosphate is affected by the rate of glomerular filtrate, an increase in filtration rate leading to an increase in phosphate reabsorption, a decrease to a decrease in reabsorption; the rate of reabsorption in terms of volume of glomerular filtrate being fairly constant, and that it is convenient to express it in terms of 100 c.c. of glomerular filtrate.

We find that in man the rate of reabsorption of phosphate expressed as P in mg./min./100 c.c. glomerular filtrate for the 62 periods is 3·63 σ 0·83; where the Inulin and Diodone were given subcutaneously—36 periods, 3·16 σ 0·68, and where they were given intravenously—26 periods, 4·02 σ 0·6.

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A simple apparatus for remote nerve stimulation in the unanaesthetized animal.

By J. Greig, *Department of Electrical Engineering, Northampton Polytechnic, London*, and A. Ritchie, *Physiology Department, University of Edinburgh*

The electrical stimulation of nerves normally inaccessible in the conscious animal may be effected by the use of induced electric currents excited from an external source.

The circuit arrangement of a simple apparatus for this purpose is shown in Fig. 1.

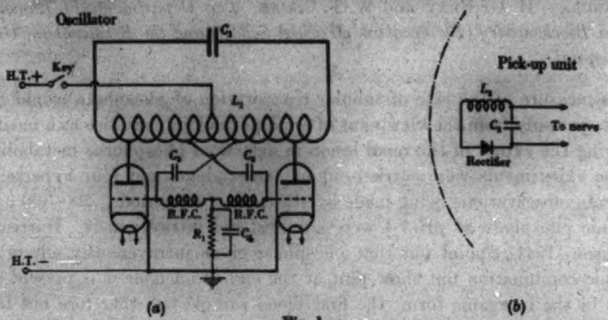

Fig. 1

The oscillator circuit, which is push-pull, operates at a frequency of about 100 kc./sec. The alternating magnetic field of the coil L_1 extends at a useful intensity to a distance of some 20 or 30 cm. This field serves to induce a high-frequency voltage in a pick-up coil L_2 which may be placed in a suitable position embedded in the tissues of the experimental animal. As the type of current required for nerve stimulation is interrupted d.c. the pick-up coil is arranged to feed a reservoir condenser C_5 through a small copper oxide rectifier. The direct voltage developed across C_5 is applied by contact points to the nerve. Interruption may be effected by a mechanical or electrical interrupter (shown for simplicity as a switch in series with the high-tension supply to the oscillator). A convenient and flexible method of interruption is to apply an additional fixed grid bias to the oscillator of sufficient value to prevent oscillation and to employ a positive pulse from a pulse generator to 'lift' this bias and permit oscillation for a suitable interval followed by the appropriate quiescent period. With this arrangement the ratio of 'on' to 'off' period is readily varied. Alternatively the oscillator may be made self-interrupting by adjustment to the 'squegging' condition, but this method, although simple, is relatively inflexible.

The size of the complete pick-up unit consisting of pick-up coil, miniature rectifier and reservoir condenser, all embedded in wax, depends to a considerable extent on the voltage required, but for normal purposes, where the requisite voltage does not exceed 0·5 V., a space some 3 cm. square by 0·5 cm. deep is adequate.

The output voltage is very considerably increased if the pick-up unit is operated at or near resonance, but this condition is necessarily much more sensitive to slight maladjustments.

PROCEEDINGS
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An explanation of the retinal direction effect. By H. HARTRIDGE.
(St Bartholomew's Medical College)

As described in detail in the communication which adjoins this one, there are two alternative plans for the trichromatic theory:

(1) Three types of cone: for red, for green and for blue.

(2) One type of cone for all colours.

Stiles found that the directional effects of the retina differed according to the colour of the light used for the measurements, thus supporting the three-cone hypothesis. It is not easy, however, to surmise the precise arrangement of the cones.

(1) Suppose the cones to be distributed irregularly with the red cones tilted one way, the green cones another way and the blue cones a third way, then here and there should be spaces between the cones. Histological sections do not support this conclusion.

(2) Suppose that one red cone, one green cone and one blue cone together form a unit and that these units make up the mosaic of the retina. Suppose further that in each of these units either the tips, or the bases, of the cones tend to approach one another, and that in each unit the red cone occupies a constant position (say on the right) and similarly for the green cone and the blue cone, then red cones everywhere will have a constant tilt in a particular direction, and similarly for the green cones and for the blue cones. But here also the inclination of the different cones should be visible in histological sections of the retina, which is not the case.

(3) Suppose there are areas of the retina occupied exclusively, or almost exclusively, by cones of a particular kind. At one place, for example, red cones predominate, at another place green cones and at a third place blue cones. When the observer is using red light he causes the image to fall on the place where the red cones predominate. These cones all lie tightly packed side by side and are, therefore, all inclined in the same direction. When, on the other hand, he is inspecting objects illuminated by green light he makes use of a retinal area where green cones predominate, and this will have a different tilt from the cones in the red area. In consequence the directional effect for the green rays will be different from that of the red rays.

(4) This supposition is acceptable, for it is a well-known aspect of the law of chance, if three populations are distributed in an haphazard manner, that there will be places where one of these populations will predominate at the expense of the other two.

The rival theories of trichromatic vision. By H. Hartridge.

(*St Bartholomew's Medical College*)

The trichromatic theory states that there are three separate mechanisms provided by the foveal cones, one responding to red rays, another to green rays and a third to blue rays. There are two different plans which might provide the required mechanisms: the single-cone plan and the triple-cone plan. According to the single-cone plan, the foveal cones are all of one type, being stimulated by all rays of the visible spectrum. According to the triple-cone plan, the foveal cones are of three different types: those for red, those for green and those for blue.

The evidence pro and con these two plans will now be considered.

(1) The study of the electrical responses of the retina by Granit, Hartline, etc., supports the three-cone plan with the possible addition of some cones for all rays (dominators) and cones for blue-green rays.

(2) The properties of nerve conduction support the three-cone plan. The single-cone plan could only work if each nerve fibre could transmit three different messages to the brain.

(3) The study of colour blindness supports either plan.

(4) The study of the peripheral retina, which is green blind, supports either plan.

(5) The retinal direction effect, as mentioned in the accompanying communication, is strongly in favour of the three-cone plan, particularly if the red cones, the green cones or the blue cones are concentrated in separate areas according to the law of chance (special triple-cone plan).

(6) Visual acuity tests support either the single-cone plan or the special triple-cone plan mentioned under (5) and referred to in the accompanying communication.

The position may be summarized as follows:

	Single-cone plan	Triple-cone plan	Special triple-cone plan
(1) Electrical responses	.	×	×
(2) Nerve conduction	.	×	×
(3) Colour blindness	×	×	×
(4) Peripheral retina	×	×	×
(5) Retinal direction effect	.	.	×
(6) Visual acuity	×	.	×

It will be seen that the special triple-cone plan is the most likely according to the evidence at present at our disposal.

An approximately linear and isotonic frontal writing lever. By H. O. Schild. (*The Pharmacology Department of University College, London*)

As the frontal writing lever rises from the horizontal, the movement of the tip of the writing point is not strictly proportional to the contraction of the tissue. At small angles the magnification (M) approximates to the ratio of lever arms l_1/l_2, otherwise it is given by

$$M=\left(\frac{l_1}{l_2}\right)\frac{A+\sin\theta-\sqrt{[A^2-(1-\cos\theta)^2]}}{B-\sqrt{[B^2-2B\sin\theta+2(1-\cos\theta)]}}.$$

If θ = angle of lever with horizontal, w = effective length of writing point, d = distance of point of attachment of tissue to horizontal lever, then $A=w/l_1$ and $B=d/l_2$; it is assumed that in the horizontal position the tip of l_2 lies vertically above the tissue and that of l_1 in the vertical plane of the drum. As the lever is tilted deviations from linearity arise owing to the writing point and muscle string deviating from vertical. The error due to the string is $<0{\cdot}5\%$ between 0° and 30° if $B>4$, that due to the writing point may be corrected to any desired extent by lengthening it (increasing A). Fig. 1 shows the error at angles between 0° and 30° of four levers differing only by the length of their writing points. An adjustable angular stop may be used to limit the excursion.

Fig. 2.

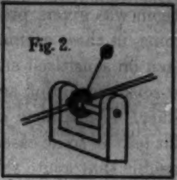

Fig. 1.

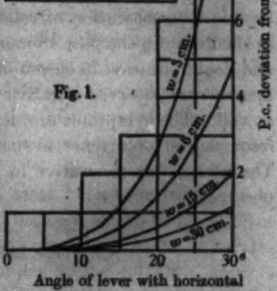

Fig. 1. Percentage deviation from linear magnification at excursions from horizontal up to +30° when $l_1=15$ cm., $l_2=3$ cm., $d=21$ cm. and $w=3, 6, 15, 30$ cm. An almost identical deviation in the opposite direction occurs between 0° and −30°. Both deviations added give the total error.

The pull exerted on the tissue by a weighted frontal writing lever often varies with the excursion. An empirical correction enabling the lever to balance in all positions against a given weight may be made by means of a pin (Fig. 2) soldered to the movable spring collar and suitably weighted and tilted. The amount of correction thus applied varies as the lever moves, the direction and rate of change of the correction being dependent on the angle the wire makes with the lever. Instead of the pin, a fine threaded rod with a nut acting as weight may be used, in which the effective counterweight depends on the distance of the nut from the pivot.

I wish to thank Dr W. H. Newton for the use of his notes on the frontal writing lever.

Anaphylactic shock in the guinea-pig. By L. B. Winter. (*Department of Physiology, University of Sheffield*)

In the course of experiments in which reacting doses of antigen have been injected into guinea-pigs sensitized by serum proteins, severe symptoms of anaphylactic shock have occurred in a number of animals; yet the uterus, one horn of which was removed under ether anaesthesia before the reacting dose of antigen was given, proved to be insensitive when tested in the bath. The symptoms, in these animals, may have been caused by the direct action of the antigen on sensitized smooth muscle in the lungs, but it is difficult to account for sensitization of smooth muscle in one situation in the body and not in another. It has been shown [Winter, 1944] that the liver may play an important part in some cases of acute anaphylactic shock in the guinea-pig, and it is possible that acute shock in animals whose uteri are insensitive may be due to sensitization of cells other than those of smooth muscle, possibly in the liver. In the past 18 months fifteen such observations have been made, and death occurred in eleven of the animals. Some indications of this phenomenon were observed in earlier work, but at that time they were not regarded as valid. The symptoms are, however, typical of anaphylactic shock and the frequency of occurrence is such that these cases can no longer be ignored. The uterus was insensitive in 15·7% of animals in which acute shock was observed.

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Renal hypertrophy. By C. Reid. (*Physiology Department, London Hospital Medical College*)

Removal of one kidney, as is well known, leads to hypertrophy of the surviving kidney, but a further increase in size can be induced in the remaining kidney within 2 weeks by a protein diet (lean meat only), a dietetic procedure which also induces hypertrophy of both kidneys in intact animals such as mice, rats and cats. These hypertrophied kidneys have about the same percentage of total N as normal kidneys, so that deposition of N must occur. A comparable increase in size of a remaining kidney does not occur after removal of the pituitary or thyroid gland.

The hypertrophy due to protein feeding is reversible when the animals are returned to their original diet with a lower protein content. Further, the growth of the kidney does not appear to be associated with the necessity to excrete increased amounts of N end products as the exhibition of urea, uric acid, creatine, creatinine and purine derivatives to normal mice does not increase kidney weight significantly. Moreover, the feeding of single amino-acids such as glycine or alanine is without comparable effect on kidney weight.

It is perhaps permissible to link the causation of hypertrophy in a remaining kidney with that in the kidneys of protein-fed animals, and to suggest that hypertrophy in both cases follows an increased supply to the organs of the constituent amino-acids of protein. It is noteworthy that the liver and the kidney both show deposition of N with high protein feeding and are also important centres for deamination.

Steroids as structurating agents on social behaviour in castrated 'Benzedrinised' mice. By M. R. A. Chance. (*Glaxo Research Laboratories, Greenford, Middlesex*)

The normal mouse of either sex treated with 20 mg. of amphetamine sulphate ('Benzedrine') per kg. body weight shows an activity slightly more marked than but similar in behaviour to that of the untreated animal.

On the other hand, such animals in groups show a characteristic behaviour towards one another differing markedly from that of untreated mice, aggressiveness and defence attitudes alternating with escape reactions during excitation with the drug.

Unlike normal animals castrates show the same behaviour in groups as when alone; there is rapid random alternation between activities of different types. Male castrates treated with desoxycorticosterone acetate or certain other steroids, prior to administration of amphetamine sulphate, show a structurated pattern of behaviour similar to that of intact animals receiving excitatory doses of amphetamine sulphate alone; castrated female mice show, under the influence of these steroids, only a slight tendency to return to the normal behaviour of amphetamine-treated males or females.

Enzymic oxidation of mescaline in the rabbit's liver.

By H. Blaschko. (*Department of Pharmacology, Oxford*)

Preparations from guinea-pig's liver containing the enzyme amine oxidase attack mescaline (β-3:4:5-trimethoxyphenylethylamine) only very slowly [Blaschko, Richter & Schlossmann, 1937; see also Pugh & Quastel, 1937], but Bernheim & Bernheim [1938] showed that similar preparations from the rabbit readily oxidized mescaline. This probably explains why the rabbit can tolerate relatively high doses of mescaline [Slotta & Müller, 1936]. Bernheim & Bernheim found that the oxidative desamination of mescaline differed from that of typical substrates of amine oxidase: the oxidation of mescaline was inhibited by cyanide. They concluded that the oxidation of mescaline requires the presence of amine oxidase and of a cyanide-sensitive factor.

Differences between the oxidation of mescaline and of *l-p*-sympatol, a typical substrate of amine oxidase, make it unlikely that the enzyme amine oxidase takes part in the oxidation of mescaline. These differences are:

(1) *sec.* octyl alcohol, a strong inhibitor of amine oxidase, did not interfere with the oxidation of mescaline.

(2) Similarly, methylene blue had only a very slight inhibitory effect on the oxidation of mescaline in concentrations in which the oxidation of sympatol was completely abolished.

(3) Adding mescaline to sympatol in saturation concentration caused an increase in the rate of oxygen uptake.

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A new method of staining non-haematin iron in erythrocytes.

By R. A. M. Case (introduced by M. E. Nutt)

Non-haematin iron may exist in erythrocytes under certain conditions in a stainable form [Grüneberg, 1941; Case, 1943; Granick, 1943]. Present methods of staining have been based on the Prussian blue reaction or the action of hydrogen sulphide on unfixed cells. The latter method is open to criticism in so far as it is possible to produce the siderocyte from a normal erythrocyte by the action of hydrogen sulphide on the unfixed cell.

The Prussian blue reaction is uncertain unless all the iron is 'unmasked' by reduction (ammonium sulphide technique of Nishimura [1910]) or oxidized (acid-alcohol technique of Macullum [1895]). The former method is lengthy and offensive, and the latter method may dissolve out some of the iron.

The following method has been elaborated from chemical considerations to overcome these objections:

Two substances are in common use for the estimation of traces of iron in biological material: $\alpha\alpha'$-dipyridyl, which forms a red compound in the presence of ferrous iron, and potassium (or ammonium) thiocyanate, which forms a red compound in the presence of ferric iron. In the presence of hydrochloric acid, $\alpha\alpha'$-dipyridyl combines with ferric thiocyanate to form a purple insoluble compound [Hill, 1930].

A mixture of equal parts of (1) a saturated solution of $\alpha\alpha'$-dipyridyl in distilled water and (2) 7% potassium thiocyanate in 1% hydrochloric acid is a colourless solution which is stable for at least a month. Blood films, fixed in methyl alcohol, immersed in this solution for 10 min., rinsed in distilled water, counterstained in Biebrich scarlet (1%) or picric acid (0·2%), show the siderocytes containing purplish granules, the morphology being superior to that of the older techniques.

If the solution is kept in a slide-jar with a tightly fitting cover, and filtered occasionally, the staining mixture may be used for bath-staining for a week or more, and will deal with several hundred slides.

The speed and cleanliness of this method make it admirable for clinical and industrial use, and the improved morphology makes counting more easy.

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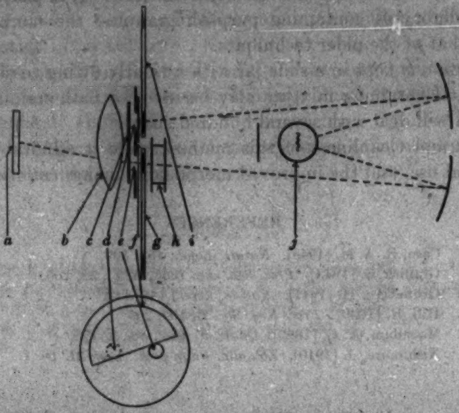
A single cell photo-electric absorptiometer in which the intensity of light source and characteristics of the photo-cell may change without causing error in estimation. By J. M. Peterson. (*The Physiology Institute, Cardiff*)

Principle. Light is reflected from a single source (*j*) through two identical apertures (*d*, *e*). The two beams of light are bent by the lens (*b*) to fall on the same area of the photo-cell (*a*). *f* is a semicircular rotating disk which alternately interrupts the two paths of light while maintaining the effective aperture constant. *g* and *i* are two annular spectroscopically neutral wedges, *g* having a scale round its margin. *h* is the substance whose absorption is measured. *c* is a colour filter. When the intensities of the two beams of light falling alternately on the photo-cell are the same, the galvanometer deflexion remains unchanged. When they differ, the galvanometer swings with the rotation of *f*, the speed of rotation being adjusted to the natural period of the galvanometer.

Procedure. With water at *h* and wedge *g* set at zero (maximum density) *i* is adjusted to give minimum swing of galvanometer. On replacing the water by the substance whose absorption is to be measured the change in optical density of *h* is shown by swinging of the galvanometer. *g* is adjusted to balance the optical densities again and reduce the galvanometer swing to its minimum. *i* is maintained constant and consequently the intensity of light falling on the photo-cell when balance is attained is independent of variation of the optical density of the substance whose absorption is being measured. Wide variations of the light intensity by means of a resistance in the lamp circuit do not interfere with the balance. The fact that the same area of the photo-cell is used in equating the optical densities on the two sides eliminates error due to change in the cell.



Accuracy. The apparatus has so far been used only for haemoglobin (alkaline haematin) estimation. Over a wide range of haemoglobin concentration the standard error, stated in terms of oxygen capacity, has been about 0·05 ml. O_2/100 ml. blood, for estimations based on five replicates.

PROCEEDINGS
OF THE
PHYSIOLOGICAL SOCIETY
3 *June* 1944

The rate of the formation of urea in normal and nephrectomized animals. By C. Reid, *Physiology Department, London Hospital Medical College*

The kidney forms ammonium ions and, incidentally, it should be noted that it has been shown to be capable of a higher rate of deamination per g. of tissue than any other tissue of the body. It is conceivable, therefore, that most of the NH_4^+ formed in the kidney is converted ordinarily into urea in the liver.

To obtain presumptive evidence of this conception the total urea formation has been estimated during 24 hr. in several series of normal and doubly nephrectomized rats, fasting for 24 hr. and weighing usually between 150 and 200 g., in the following way:

(1) Determination of the volume of body fluids in equilibrium with urea. Let x = volume of body fluids in ml. a = mg. urea per ml. blood before injection. b = mg. urea per ml. blood 5–10 min. after the injection of 250 mg. urea into a vein (1 ml. of a 25% solution).

Then
$$ax+250=(x+1)\,b.$$

The volume of the body fluids in equilibrium with urea appeared to be of the order of 40–50 ml. per 100 g. rat.

(2) Rats fasting for 24 hr. were nephrectomized under nembutal and then kept for a further 24 hr. when the total urea formation was computed from the blood urea concentration and the calculated volume of the body fluids (vide (1) supra). Control series of rats were given the same treatment without nephrectomy. Their total urea production was found by adding the urine urea to the body fluid urea.

Result. The total urea formed (mg. per 100 g. rat) in the nephrectomized rats was about one-third to one-quarter less than that in intact animals. This result suggests that of the urea, which we know is formed exclusively in the liver, about one-third to one-quarter may be derived from deaminating processes of the kidney.

PROCEEDINGS
OF THE
PHYSIOLOGICAL SOCIETY
29 *July* 1944

Action of drugs on bleeding time. By G. Ungar. *Nuffield Institute for Medical Research, Oxford*

The action of a number of drugs was studied on bleeding time in guinea-pigs. Each ear was pricked with a needle (1·mm. diameter), and bleeding was recorded by means of a blotting paper. Bleeding was considered stopped when no issue of blood or any fluid could be detected for 10 sec.

In eighty-eight measurements made in forty-four control guinea-pigs the mean bleeding time was 130 sec. ±3. There was no significant difference according to sex or age.

Chlorazol-fast pink, used as an anticoagulant, did not lengthen the bleeding time, but this was increased by histamine and acetylcholine. By increasing the dose of the latter drug above 0·05 mg./kg. a reversal of the effect was observed, due presumably to the release of choline which had a definite shortening effect on the bleeding time.

Bleeding time was shortened by the pressor principle of the posterior lobe of the pituitary (Pitressin), but not by adrenaline or ephedrine. This supports the current view that the latter substances have no action on the capillaries.

Another group of substances was found to shorten the bleeding time without having any vasoconstrictor effect; they were a whole adrenal cortical extract prepared by Kendall (desoxycorticosterone acetate had no action), the corticotrophic hormone of the pituitary, ascorbic acid and vitamin P.

The maximum decrease observed was 45 %. In groups of eight measurements the standard error of the mean was about 10 %. The change in bleeding time induced by drugs was in linear relation with the logarithm of the dose. This shows that bleeding time is suitable for pharmacological investigation and could be used as a method of standardizing some of the drugs mentioned above.

The effect of trauma on bleeding time and capillary resistance. By G. Ungar. *Nuffield Institute for Medical Research, Oxford*

Fleisch & Posternak [1943] observed a shortening of the bleeding time in rabbits following numerous punctures performed on the same ear and attributed it to the effect of trauma. Their experiments were repeated in guinea-pigs traumatized according to a standard method [Ungar, 1943] and their interpretation was confirmed. In eighty-eight measurements in normal guinea-pigs the bleeding time was 130 sec. ± 3. After an interval of 6 hr. the bleeding time dropped to 87 sec. ± 9. After 3 days it was 78 sec. ± 5, and returned to the normal figure of 128 sec. ± 10 after 8 days. (The latter figures represent means of sixteen measurements.)

At the same time, there was an increase of the capillary resistance as measured by the negative pressure method on the ear of guinea-pigs. In twenty-five control animals the mean capillary resistance was 245 mm. Hg ± 9. After trauma, it went up to 331 mm. ± 18 (6 hr. after) and to 387 mm. ± 28 (3 days after). After 8 days it returned to 249 mm. ± 15. These figures are means of eight observations.

It has been observed, moreover, that blood serum of traumatized guinea-pigs, when injected into normal animals, shortens the bleeding time and increases the capillary resistance. Normal guinea-pig serum has only a slight effect in increasing the bleeding time. It was previously suggested that blood of traumatized guinea-pigs contains a substance identical with the corticotrophic hormone of the pituitary [Ungar, 1944] which also shortens the bleeding time and increases the capillary resistance.

The simultaneous changes in bleeding time and capillary resistance suggest that they both depend on some common factor acting on the capillary wall.

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The rate of mixing and disappearance of T. 1824 (Evans blue) injected into the circulation.

By E. W. H. Cruickshank and I. C. Whitfield. *Department of Physiology, Marischal College, Aberdeen*

In the determination of dye disappearance curves it has been assumed that (1) the initial rapid fall in concentration (6 min.) is due to mixing, (2) the subsequent portion of the curve, in which the fall in concentration is constant, is due to disappearance of the dye, and (3) the tangent point of the two curves represents the time at which mixing is complete [Gibson & Evans, 1937; Kennedy & Millikan, 1938].

In the cat we have found that mixing is complete within one minute of dye injection. This was shown by taking samples from jugular and femoral veins, at 15, 30 and 60 sec. and thereafter at longer intervals, the dye concentrations becoming equal between 30 and 60 sec. (Fig. 1). The initial rapid fall in the curve, however, continues for 5–6 min., the curve then becoming virtually linear. If now a second injection of dye be made, the rapid component is absent from the new curve. Evidently the dye is initially taken up by some mechanism which becomes saturated more or less rapidly in different animals [cf. Courtice, 1943]. Previous blocking of the reticulo-endothelial system with Indian ink also abolishes the rapid phase (Fig. 2). Since the rapid fall in the curve is not due to mixing, plasma volumes based on plotting the disappearance curve from 6 min. onwards and extrapolating to zero time are, at usual dye concentrations, liable to an error which may exceed 20 %. By injecting a previous 'saturating dose' of dye and determining the disappearance curve after a second injection, a true value for the initial concentration of dye may be obtained by extrapolation.

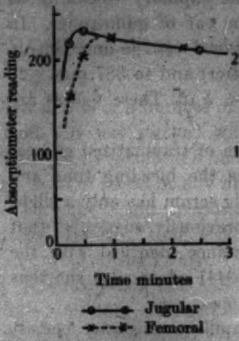

Fig. 1.

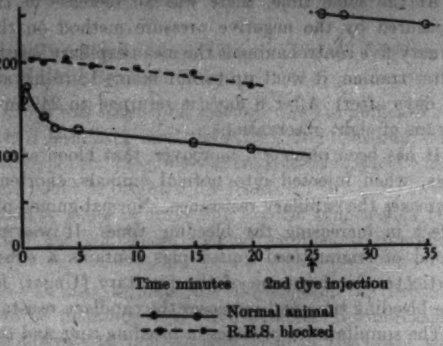

Fig. 2.

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Urinary changes during water diuresis.

By J. A. Barclay and Marjorie E. Nutt. *Department of Physiology, University of Birmingham*

Various workers [Marshall, 1920; Eggleton, 1943] have investigated the effects of water diuresis on the composition and reaction of the urine. The conflicting results reported may derive from the fact that experiments have been performed on relatively small groups of subjects. In the following experiments, although the numbers are not large, results show all the variations in chloride excretion previously described.

Ten subjects took no breakfast, but drank 200 c.c. of fluid on rising; nine subjects ate their usual breakfast. 1½–2 hr. later the bladder was emptied and two samples of urine collected at 15 min. intervals. 800 c.c. of water was drunk and urine collected every 15 min. until the diuresis was over. The volume of urine passed during the diuresis averaged 711 c.c. in eighteen subjects, but varied from 191 to 1174 c.c. In one subject 1300 c.c. of fluid failed to produce a diuresis. The taking of a meal did not influence the results.

Results indicate that the movement of urinary *p*H is not towards the *p*H of the blood but towards some lower value, since all urines from *p*H 6·9 upwards showed a fall during diuresis and more acid urines showed a rise. That the *p*H is not entirely dependent on volume changes is shown by the fact that in eleven control experiments, in which no water was taken, *p*H changes occurred when the urine volume was constant; subjects with higher *p*H values showed a slight fall, and those with lower values a slight rise, followed by a slight fall, during the morning.

In control experiments the concentration of chloride remained constant, and the curves for total chloride excreted mirrored the volume curves. Diuresis produced varying results. In three subjects there was a fall in chloride excretion; in three a slight rise followed by a fall; in one a prolonged slight rise; in three a sharp rise followed by a fall, but not to the original level; and in five a rise in the chloride excretion which mirrored the increased urine volume.

It is suggested that the varying amounts of fluid passed during diuresis are expressions of the state of hydration of the individuals. The subject who drank 1300 c.c. without producing a diuresis seldom feels thirsty and normally drinks very little. When a diuresis was produced his chloride output fell, a finding reported by McCance & Young [1944] in rehydration of dehydrated persons.

The variations in chloride excretion during diuresis may depend on the different concentration of chloride in the individuals' body fluids. The results in general confirm McCance & Young's view that the excretions of water and chloride are closely interrelated.

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The effect of exercise on the composition of the urine. By J. A. Barclay and Marjorie E. Nutt. *Department of Physiology, The University of Birmingham*

During a class experiment in October 1943, it was found that the effect of exercise on water diuresis differed somewhat from the results reported by Eggleton [1942]. Exercise produced no inhibition in four subjects, slight inhibition in nine, moderate inhibition in seven, and marked inhibition in only five. These experiments were performed in the afternoon.

The present series of experiments, performed in the mornings, produced inhibition in all but one of thirty trials. Thirteen experiments were performed after a normal breakfast, and seventeen on subjects who had taken a cup of tea, but no food. 800 c.c. of water was drunk, and the volume of fluid passed during the diuresis, including the period of exercise and recovery, averaged 597 c.c. as compared with 711 c.c. when no exercise was taken. Breakfast did not influence the results. The degree of inhibition was greater when the diuresis was smaller. In three cases there was no re-establishment of the diuresis after exercise. Measurement of the subjects' fluid intake and output during the next 24 hr. gave no indication of a greatly prolonged inhibition.

Exercise caused a sharp fall in urinary *p*H which in most cases outstripped the fall in volume. The *p*H began to rise again half an hour after exercise, even when the volume was still falling; *p*H continued to rise as the diuresis reappeared, and then fell. In all subjects the total chloride output fell sharply after exercise. This change, too, was sometimes apparent before the volume change, a finding evident in the results of Wilson, Long, Thompson & Thurlow [1925]. After about an hour, chloride excretion rose again in those subjects who showed an increased chloride excretion during water diuresis. In the others it remained low.

The time of occurrence of maximum inhibition, i.e. ½–1 hr. after the exercise, makes it unlikely that renal vasoconstriction is the cause of the fall in urine volume. It is suggested that water is retained in order to combat the rise in osmotic pressure of the blood which occurs after exercise [Margaria, 1930].

The degree to which a water diuresis is inhibited by exercise may depend on the state of hydration of the subject. In a well-hydrated individual the increase in osmotic pressure of the blood may not suffice to counteract the diluting effect of the water drunk and the inhibition may be slight or non-existent. The greater percentage of successful inhibitions in the morning experiments may indicate that individuals tend to be less well hydrated then than in the afternoon.

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Staining of the granules of leucocytes with 2, 6-dichlor-phenol-indophenol. By F. Jacoby. *Department of Physiology, The Medical School, Birmingham*

Malowen [1939] observed that the leucoform of 2, 6-dichlor-phenol-indophenol (DCPIP) changes to a deep red in the presence of horse-radish peroxidase and

H_2O_2. In applying this reaction to human blood films for the staining of the granules of leucocytes I found that the use of the unreduced blue reagent gives excellent results and simplifies the technique (see separate printed communication). The discovery of this reaction, as a very suitable one for staining the leucocytic granules, and its detailed investigation led to a number of observations worth recording.

DCPIP (unreduced) + *dilute* H_2O_2. Granules stain deep purple. The dye can be employed in a solution of 0·01 g. % and higher. In lower concentrations the reaction becomes less intense and soon negative. A 0·05 g. % solution will give unfailing results on fresh films. The eosinophils are not only the first to react and their large granules the ones to stain most heavily, but their peroxidase is also resistant towards various noxious influences, such as KCN, excess of H_2O_2 and dry and moist heat, which completely inhibit the reaction in neutrophils. Longer exposure to these reagents or higher concentrations will abolish the purple reaction also in the eosinophils. Some blood films, several years old, gave a strong positive reaction in all eosinophils whilst every neutrophil was negative. Previous treatment with Lugol's solution for 5 min., which in itself has no visible effect on the granules, causes them, particularly in neutrophils, to be stained a deep red instead of purple.

DCPIP (unreduced) *without* H_2O_2. In eosinophils discrete blue granules always appear. Each granule probably corresponds to the centre of one of the characteristic eosinophil granules, since subsequent treatment with the dye + H_2O_2 produces a dark purple ring round each granule of about the same size as an eosinophil granule. Neutrophils show only minute pale blue granules, or none at all, so that the stain is more or less selective for the eosinophils. It can be intensified by heat (130° C. for 25 min.), especially on films which are a few days old, and the blue granules then become a deep sky-blue. The blue stain is not permanent.

Leucoform of DCPIP (reduced by adding a few drops of strong solution of vitamin C) + *dilute* H_2O_2. Granules stain deep purple. The only advantage of employing the leucoform is that the reaction can be easily watched under the microscope. Staining starts almost immediately, particularly in eosinophils, and each granulocyte acts as a centre of oxidation, turning the reagent into its coloured (blue) form which then slightly stains the surrounding red cells.

Leucoform of DCPIP without H_2O_2. Granules stain blue as with the non-reduced form. This seems to indicate that the blue staining of the granules is not due to adsorption alone, but involves an oxidative process.

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Recognition of renal calculi by chemical methods and X-ray diffraction patterns.

By J. A. Barclay, *Physiology Department, University of Birmingham*; W. T. Cooke, *United Hospital, Edgbaston, Birmingham*, and M. Stacey, *Chemistry Department, University of Birmingham*. With assistance in the X-ray patterns by Dr A. D. Booth, and chemical analysis by Mr P. W. Kent.

Examination of more than thirty stones has so far revealed five types. The majority, however, fall into two main groups which have quite distinct X-ray diffraction patterns. In the rarer groups there is some degree of overlapping.

The characteristics are as follows:

Most stones, including those of type D, contain magnesium phosphate in small amounts.

Type B. The carbonate-apatite group

To this group belong most stones in the bladder, kidney and salivary gland. The X-ray diffraction pattern is that of the mineral dahllite, and furthermore is identical with that of teeth and bone.

Chemically, a type B stone can readily be recognized by its immediate effervescence with dilute HCl and by its high phosphate content. Some type B kidney stones contain 2–5 % cholestrol.

Type B^1. Hydroxy-apatite stones (rare)

These give a closely related diffraction pattern, but give little or no effervescence with acids.

Type C. The calcium oxalate stone (? Whewellite)

This is a common type of kidney stone containing a high percentage of calcium oxalate with small amounts of phosphate. It is insoluble in dilute HCl and gives no CO_2.

Type A

Type A is an oxalate stone free from phosphate and quite distinct from type C. There was only one example of this type from the bright green pigmented centre of a large type B bladder stone.

Type D

In this class we put the stones which are almost entirely organic, e.g. cystine stones. Some type D stones give patterns typical of fibres.

The X-ray pattern gives a rapid means of detecting the *type*, but does not detect the actual chemical constitution.

A new peroxidase reaction on leucocytes employing 2, 6-dichlor-phenol-indophenol. By F. Jacoby. *Department of Physiology, The Medical School, Birmingham*

Reagents necessary

(1) Conc. formol, (2) absolute alcohol, (3) 0·05 % aqueous solution of 2, 6-dichlor-phenol-indophenol (can be stored in an ice-box for at least a few months), (4) H_2O_2, about 4 (!) vol. %.

Technical procedure

(1) Fix the freshly prepared air-dried blood film in a mixture of 1 pt. formol and 9 pt. absolute alcohol. 2–3 min.

(2) Wash in water.

(3) Treat film with solution of DCPIP, to every 5 c.c. of which 4 drops of H_2O_2 had been added prior to use (this mixture does not keep beyond a few days). 3–5 min.

(4) Wash in water, blot film dry and examine with oil immersion lens. If mounting is desired, neutral balsam should be used.

Result

The 'peroxidase-positive' granules of the granular leucocytes are stained a deep purple-violet, most heavily in the eosinophils whose large granules often show a dark purplish peripheral ring and a lighter interior. Some of the monocytes may appear as if lightly sprinkled with fine granules, but others and all the lymphocytes are negative. Nuclear areas are left completely unstained. The erythrocytes—if the film was dried quickly—will be greenish blue; otherwise they will have lost the stain. But prolonged washing does not affect the stain of the granules, which is permanent. They are shown up with extreme sharpness, clarity and precision. This makes this method superior to Graham's benzidine reaction, where often the whole cytoplasm is diffusely stained yellowish brown, and also to Sato's benzidine reaction, where frequently various bluish crystals and granules are precipitated on the film and the red cells are but badly preserved.

Counterstain

As a mere nuclear counterstain neutral red (0·5% sol.) has given the best result in the way of contrast, particularly if the erythrocytes have kept a bluish colour. It can be applied concomitantly with or subsequently to step (3). Methyl green, too, can be recommended. The reaction can also be followed by Leishman's or related blood stains, though longer staining times than usual are necessary. With these counterstains the granules appear almost black. If Delafield's hematoxylin is used, the colour of the granules turns into a brownish red.

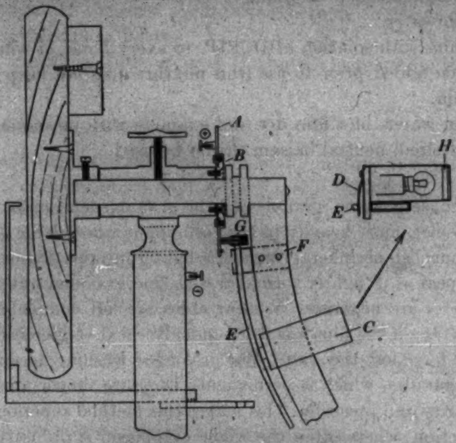
The technique has been successfully applied also to bone marrow films and to frozen sections of formalin-fixed tissue. In the latter much longer reaction time in the DCPIP has to be allowed.

A modified Priestley-Smith perimeter. By K. A. Webb.

Physiology Department, University of Birmingham

The bits of coloured and white paper used in the original perimeter do not reflect light of uniform intensity and rapidly become dirty and worn. These difficulties may be avoided by substituting a point source of light with suitable filters as follows (see Figure):

A circular brass disk *A* is riveted on to a fibre washer *B* which is screwed into the main bearing, thus insulating it; the disk is connected to one pole of a battery. The other pole of the battery is connected through the main upright to the rotating arm. The lamp housing *C* consists of a metal box $\frac{7}{8}$ in. × $\frac{7}{8}$ in. × $1\frac{3}{8}$ in. fitted so that it slides on the rotating arm. The back of the lamp housing, suitably insulated, has a spring contact *D* which presses on $\frac{1}{8}$ in. brass rod *E*, suitably bent, fixed to the rotating arm by vulcanite insulators *F*. To the central end of the brass rod is fixed a sprung nipple *G* (one of the nipples from a bayonet-type lamp-holder). This enables contact to be made through the complete arc of the circle.



A modified Priestley-Smith perimeter.

The light source is 3·5 V. bulb which shines through a $\frac{1}{32}$ in. hole in the face of the housing. A slot *H* is cut on one side of the lamp housing to carry filters.

Filters can be made by dyeing cellophane red, green and blue and mounting between glass. The light absorption by the filters was adjusted by (*a*) time in dye and (*b*) concentration of the dyes. A photo-electric cell was used to measure the light transmitted.

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30 *September* 1944

The inositol content of the mammalian heart. By L. B. WINTER.

Department of Physiology, University of Sheffield

Although the glycogen content of the different tissues of the heart, both mammalian and cold-blooded, has been studied by numerous workers, no investigation appears to have been made as to whether there is a differential distribution of inositol. Ox hearts were used, brought to the laboratory as soon as possible after the animal was killed, but not cooled, since it was assumed that post-mortem changes would affect the tissues equally. In each experiment, 18 g. of tissue were taken, as free as possible from visible fat. The tissue was dissolved by reflux with potash for 2¼ hr. and the inositol was estimated by crystallization and weighing [Winter, 1940]. It was found that, although there were considerable differences in inositol content between different hearts, the ventricle always contained more inositol than the auricle of the same heart. The differences are shown by the following figures:

Auricle mg. inositol per 100 g.	Ventricle mg. inositol per 100 g.
77·1	113·5
68·9	85·7
92·2	134·8
84·8	99·8

Estimation of the inositol content of Purkinje tissue has been attempted. Hearts were used which showed a well-defined bundle of His. 12 g. of muscle were cut out, containing as much of the bundle as possible, and 12 g. from the inner wall of the ventricle, free from visible Purkinje tissue. Eight experiments were performed. In every case the muscle with the bundle of His contained less inositol than that from another part of the same ventricle. Direct estimation of the inositol was carried out on 8·6 g. of Purkinje tissue obtained by dissection from twenty-nine hearts in the course of 3 weeks; each small batch was placed in the same flask with the addition of the required amount of potash and was left at +3° C. until the last addition, when the whole was refluxed. The inositol content was 53 mg./100 g. Though the Purkinje fibres are unlikely to contain more inositol than the ventricular muscle, it cannot yet be said with certainty that they contain less, since it is difficult to free the fibres from connective tissue with which they are intimately bound up. Davies & Francis [1941] have drawn attention to this source of error in the case of glycogen estimation in the frog's heart.

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The effect of adenosine triphosphate on a water diuresis in man.

By H. N. Green and H. B. Stoner. *Department of Pathology, The University, Sheffield*

The effect of the intravenous injection of the magnesium salt of adenosine triphosphate (ATP) on a water diuresis was observed in three normal male subjects. When ATP was given at the same time as a litre of warm water there was no delay in the onset of diuresis, but its degree was significantly reduced. About 45 min. after the injection certain symptoms appeared. The subject felt cold with general malaise, headache and shivering. This was succeeded by a sensation of warmth and sleepiness. At this stage there was a rise in oral temperature as high as 102° F., persisting as long as 9¾ hr. The dose of ATP required to produce the effect varied from 0·25 to 1·78 mg./kg. body wt.

Under the conditions in which the subjects were living the urine was frequently alkaline. When it was acidified with NH_4Cl (6 g. daily by mouth) the effective dose of ATP was reduced—in the most resistant subject by one-third.

If it should prove that ATP plays a part in the shock syndrome, then these observations would throw some light on the nature of traumatic oliguria and aseptic traumatic fever. After severe trauma in human subjects the urine is always acid, and in a series of 109 cases we have found that the intensity of the oliguria varies with the degree of muscle damage.

The elucidation of the findings is still in its preliminary stages, but it seems probable that they represent a pre-renal deviation of water. Apart from their relation to shock, the findings seem to bear on the relationship between the heat-regulating mechanism and the water distribution of the body.

Acetylcholine formation in cell-free extracts from brain.

By W. Feldberg and T. Mann.* *Physiological Laboratory and the Molteno Institute, Cambridge*

Synthesis of acetylcholine has been shown to occur in brain slices and pulp [Quastel, Tennenbaum & Wheatley, 1936; Stedman & Stedman, 1937] as well as in brain tissue which had been dried in a desiccator and powdered before use [Feldberg, 1944]. In all these instances the synthesis took place aerobically and depended on the presence of some particulate matter, though not necessarily on that of intact cells. Recently Nachmansohn & Machado [1943] have shown that acetylcholine is formed *anaerobically* in homogenized brain tissue provided that adenosine triphosphate (ATP), fluoride, eserine and choline are added. This we were able to confirm. At the same time, however, we noticed that very little acetylcholine was formed aerobically, in spite of its known aerobic formation in brain slices and pulp. In order to explain this discrepancy, we carried out a number of experiments, the main results of which are as follows:

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(1) The enzyme system which catalyses the formation of acetylcholine in the presence of ATP has been obtained in the form of cell-free solutions, prepared by extraction of acetone-dried and powdered rat's brain. In 1 hr., at 37° C., 140–400μg./g. acetylcholine was formed, which corresponds to 500–1430μg./g. dry material contained in the extract.

(2) Only about half this amount of acetylcholine was formed aerobically. However, reduced glutathione or cysteine brought the aerobic synthesis of acetylcholine to almost the same level as that observed anaerobically. Oxidized glutathione or cystine, on the other hand, inhibited the synthesis of acetylcholine in air as well as in nitrogen.

(3) Unlike the homogenized brain tissue, the extracts prepared from the acetone powder only very slowly liberated ortho-phosphate from ATP. The rate at which free phosphate was split off remained the same in the presence and in the absence of oxygen and was not affected by the addition of choline.

(4) The function of ATP has been found to be specific. ATP could not be replaced by either adenosine diphosphate, inosine triphosphate or adenylate.

(5) Glucose, fructose and certain phosphorylated sugar derivatives inhibited the formation of acetylcholine. This could be explained by an esterification of the labile phosphate groups of ATP.

(6) The formation of acetylcholine was enhanced by K-ions and diminished by Ca-ions. Ca-ions inhibited, but K-ions did not affect the liberation of inorganic phosphate from ATP.

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Toxic extract from blood plasma of shocked animals. By H. N. Green and H. B. Stoner. *Department of Pathology, The University, Sheffield*

Green & Stoner [1944] found that the injection of magnesium salts increased the sensitivity of animals to the shock-inducing action of adenosine triphosphate (ATP) and related compounds containing a purine-pentose linkage. Magnesium-treated animals were also more sensitive than untreated animals to a variety of shock-inducing measures. Hitherto we were not able to detect with any certainty the presence of substances toxic to the whole animal in the blood or lymph from animals in shock. Using the magnesium-treated mouse as indicator, positive results have now been obtained.

Blood plasma from rats and rabbits in ischaemic shock, and from rats in glucose dehydration shock, was mixed without delay with acetone. After filtration, the residue was washed with acetone and ether- and air-dried. The residue was then extracted with normal saline, boiled, and the filtrate from this used for animal injections. This procedure corresponds to the first stage in the method used by Bielschowsky & Green [1943] to prepare ATP from striated muscle.

On injection into Mg-treated (0·5–0·75 mg. Mg/10 g. body wt.) mice, most animals were quickly prostrated by the 'shock plasma' extract, and unaffected by equivalent amounts of extract from normal plasma. The effective dose has varied with a range equivalent to 1–3 c.c. 'shock plasma'/10 g. mouse.

A plasma of greater potency was obtained from the shocked animal when the animal was injected with magnesium, and bled, when prostrate. Under these conditions as little as 0·5 c.c. plasma/10 g. mouse has proved lethal to the Mg-treated mouse.

These facts suggest that the toxic factor in 'shock plasma' may be ATP or related compounds. The more potent plasmas had an action equivalent to that of 1–2 mg. ATP/c.c. Preliminary chemical studies do not indicate that ATP, or its metabolites, is present in such large amounts and we therefore reserve judgment on the nature of the toxic factor(s) in 'shock plasma'.

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Excitatory and inhibitory processes in the synthesis of the sensations of colour and of white. By GUSTAF F. GÖTHLIN, *Uppsala* (introduced by H. E. ROAF)

Sensations of colour require three sets of nerve impulses, each set corresponding to one 'fundamental' colour. The 'fundamental' colours, when at levels sufficiently high to produce a coloured sensation, inhibit the effect of impulses which would produce the complementary colour.

Red and green are two of the 'fundamental' colours. By the following method, the quality of the third 'fundamental' colour was found to be blue. Using wave-lengths from 420 to 460 mμ and gradually increasing the intensity of the radiation, the subject was asked to name the colours as they appeared. Following a photochromatic interval he saw the first recognizable colour as blue. The red elements in the colour did not appear until higher intensity levels were reached. These observations suggest that the 'fundamental' colour is blue and that violet is a compound in which a red element is added to the 'fundamental' blue.

Colour synthesis takes place in two stages. 'Fundamental' red and 'fundamental' green when of appropriate intensities balance each other producing a sensation of yellow. If either red or green predominates, the yellow is tinted with red or green. If the intensity of yellow balances that of blue, a colourless or white sensation results. If the yellow and blue do not balance, the corresponding colour will be seen. A lack of balance at both stages will produce various colours, e.g. if the red preponderates at the first stage and blue at the second, a sensation of violet will be produced.

There are three points to be noted. (1) Inhibition is an important factor in the recognition of colour. (2) The third 'fundamental' colour is blue. (3) A mechanical analogy of two balances, the second suspended from one arm of the first, can be used to explain the effects of the combination of colours.

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Lipids in maternal and foetal blood-plasma of sheep.

By J. Barcroft and G. Popják.* *A.R.C. Unit of Animal Physiology, Cambridge; and Department of Pathology, St Thomas's Hospital Medical School, London*

The lipid fractions in maternal and foetal sheep plasmata were determined at various stages of pregnancy. Sheep may be taken as typical of forms with syndesmochorial placentas. The results cannot necessarily be applied to other mammals. The foetuses were delivered by caesarean section and blood was obtained from the umbilical cord. 3 mg. of potassium oxalate per ml. of blood were used as anticoagulant. Phospholipids, total fatty acids and non-phospholipid fatty acids (fatty acids of neutral fats and cholesteryl esters) were determined by the methods of Bloor (1928, 1929) and cholesterol by the method of Popják (1943). The results presented in Fig. 1 show that the lipids in the foetal plasma follow, more or less, and at a lower level, those in the maternal plasma. Their concentration is commonly about one-third to two-thirds of the maternal values. The close relationship between maternal and foetal plasma lipids suggests, but does not prove, that the lipids pass through the placenta and are disposed of in the foetus either by storage or otherwise at

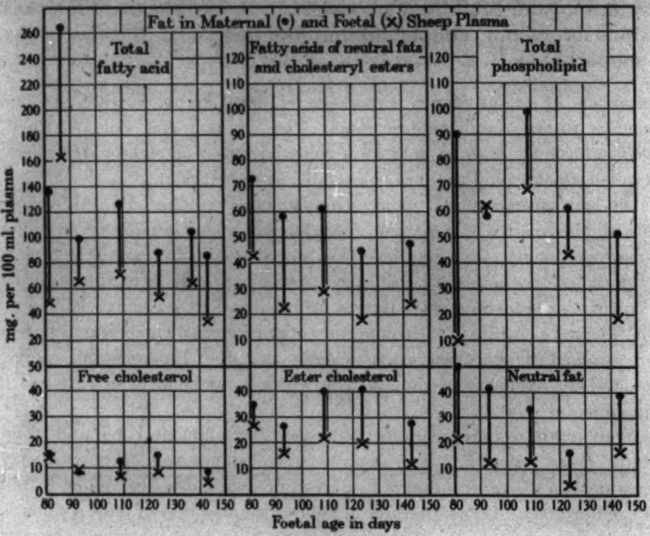

Fig. 1.

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* Beit Memorial Research Fellow.

a considerable rate. The cause of the variations observed in the lipid content of the plasma, both foetal and maternal, demands investigation by controlled experiments.

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The stimulating action of acetylcholine on the heart.

By R. J. S. McDowall. *King's College, London*

If a dose of acetylcholine is added to Ringer's solution perfusing a cat's heart, the effect depends on the dose. If the concentration reaching the heart is more than 1 in 1,000,000 the typical slowing is seen, but this is followed by a marked increase in the force of the beat which may or may not be associated with an increased rate. The duration of the increased activity varies in different hearts but may be very prolonged and bears no relation to the amount of the slowing. The stimulative stage is associated with an increased sensitivity to adrenaline, a reduced response to acetylcholine and a tending to extra-systoles.

Both the stimulation and depression are abolished by atropine, but the depressor effect is sooner affected by a small dose of atropine so that marked increases of force with large doses of acetylcholine may occur without any slowing. The stimulation is abolished by ergotoxine.

Very minute doses show a stimulation only, and a study of increasing sizes of dose suggests that the early stimulation is of the same nature as the late stimulation after large doses. Stimulation is associated with increased coronary flow and slowing with reduced flow.

Similar results have been obtained also on the heart of the frog, the rabbit and the rat.

The visibility of blue and yellow.

By H. Hartridge.
Medical College, St Bartholomew's Hospital, E.C. 1

Experiment shows that a pattern consisting of alternate blue bars and yellow bars is seen under certain circumstances as a pattern consisting of alternate black bars and white bars. Some quantitative measurements have been made on the effects of the following factors: (1) size of pattern, (2) size of pupil, (3) intensity of illumination.

Of these three variables, size of pattern has the greatest effect, and intensity of illumination the least effect.

TABLE 1. Effect of size of pattern

Observations made at 3000 ft.c. and 1 mm. pupil

Visual angle min. of arc	Blue was seen as	Yellow was seen as
8	Strong blue	Yellow
7	Blue	Pale yellow
6	Blackish blue	Cream
5	Bluish black	White
4	Black	White

An increase in pupil diameter from 1 to 3 mm. increases the probability that the blue and yellow pattern will be correctly seen. Increase in illumination from 1 to 3000 ft.c. produces a similar result. A possible explanation of the phenomenon is as follows: Owing to chromatic aberration of the eye, the blue rays form diffuse halos when the yellow rays are forming sharply focused images in the retina. In consequence, the yellow images fall on retinal areas which are already illuminated by the diffuse blue halos. Since blue rays are complementary in colour to yellow rays, the superposition of the blue rays causes the yellow rays to be greatly diluted by white light. Further, this dilution increases as the pattern gets smaller, thus making the confusion of the yellow bars with white bars increasingly likely.

In the case of the blue bars it would appear that it is not so much dilution by white light as loss of light intensity which causes them to be confused with black as the pattern becomes smaller. The progressive loss of light intensity is shown in Table 2.

TABLE 2. Effect of visual angle on loss of intensity of blue rays

Visual angle min. of arc	Percentage loss of intensity of blue rays	Visual angle min. of arc	Percentage loss of intensity of blue rays
24	5	3	40
12	10	1·5	63
6	20		

The importance of taste and smell in nutrition. By H. HARTRIDGE.

Medical College, St Bartholomew's Hospital, E.C. 1

Animals in a wild state under the guidance of their instincts, seem to be very successful in avoiding nutritional deficiencies. The question which presents itself concerns the precise mechanism of this instinct. Is the instinct to eat and to drink similarly due to the activity of chemoreceptors, and if so what is their nature and where are they situated? That the receptors for thirst are present in the mouth and throat is suggested by the fact that this sensation is largely alleviated when these structures are moistened with water, or sprayed with a solution of cocaine. Two facts suggest the possibility that the chemoreceptors for hunger are the taste buds of the tongue, possibly aided by the olfactory mucous membrane:

(1) On injecting a solution of decholin into the blood stream the subject suddenly perceives a bitter taste, so that taste buds can react not only to substances placed in the mouth, but can also respond to substances present in the blood stream.

The possibility suggests itself that the decholin may have been secreted by the glands of the mouth and tongue into the mouth cavity to reach the taste buds in the usual way. But this view is rendered unlikely from the very short latent period between the arrival of the drug at the tongue and its perception.

Another point in favour of blood stream stimulation is the extraordinary sharpness with which the perception occurs, and the equal sharpness with which it disappears as the drug is swept away again in the blood stream.

(2) In disease of the adrenal cortex in which the sodium chloride concentration of the blood decreases, there is an enhanced susceptibility of the taste buds to dilute sodium chloride solutions placed in the mouth. It is more than likely that the same thing happens in patients suffering from miners' and stokers' cramp, and that this is why these patients select foods with a high salt content. These facts seem to point to the conclusion that the taste buds of the tongue react not only to substances placed in the mouth but also to substances circulating in the blood.

A possible mechanism is that the taste-buds average the concentrations of substances in the blood and in the mouth. When a substance is deficient in the blood then its amount in the mouth must be increased in order that the concentration in the taste-buds shall remain constant.

The effect of breathing pure oxygen on the nitrogen dissolved in the cerebro-spinal fluid.

By B. McArdle (introduced by E. A. Carmichael). *National Hospital for Nervous Diseases, Queen Square, W.C.*1

The value of breathing pure oxygen to prevent 'bends' depends upon the rate at which nitrogen is eliminated. The lumbar cerebro-spinal fluid of young and middle-aged subjects of both sexes was collected over mercury in a special pipette designed for the purpose, in such a way that at no time did

Table 1

Subject	Length of O_2 inhalation in min.	N_2 in c.s.f. vol. %	Subject	Length of O_2 inhalation in min.	N_2 in c.s.f. vol. %
1	—	0·896	7	40	0·467
2	—	0·933	8	62	0·381
3	—	0·906	9	75	0·319
4	—	0·898	10	100	0·236
Average	—	0·908	11	120	0·299
5	16	0·806	12	185	0·136
6	18	0·668			

it come into contact with the air. The dissolved N_2 was determined by the method and using all the precautions of Van Slyke, Dillon and Margaria, except that 5 c.c. instead of 15 c.c. samples were used. Preliminary determinations of the Bunsen solubility coefficient of N_2 in water using 5 c.c. samples had given results agreeing very closely with those of Van Slyke. Eight subjects inhaled pure oxygen for varying periods.

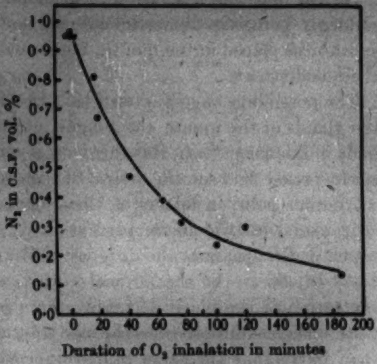

Fig. 1. The c.s.f. N_2 content of subjects breathing pure O_2.

Results. These are given in Table 1 and in Fig. 1.

The figures obtained showed the rate of elimination to follow a curve which, if extrapolated, would suggest completion in about 6 hr. Behnke, by following the N_2 content of the expired air in subjects breathing pure O_2, was able, provided he made certain assumptions, to plot curves for the rate of N_2 elimination from the water and the fat of the body. A comparison of the cerebro-spinal fluid elimination curve with those of Behnke shows that the N_2 in the cerebro-spinal fluid is relatively slowly eliminated though not as slowly as from fat. It is assumed that this is due to the relatively small area in which cerebro-spinal fluid is in contact with vascular structures.

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The diuretic response of men working in hot and humid conditions.

By J. S. Weiner (introduced by E. A. Carmichael). *Medical Research Council, Neurological Research Unit, National Hospital, Queen Square, W.C.* 1

Ten young men, aged 20–35 years, did the following work in ordinary room conditions and also at 100° F. dry bulb, 93° F. wet bulb (r.h. 77 %, eff. temp. 94° F.): after a drink of 750 c.c. of water, 5 min. step-climbing up and down a stool 1 ft. high 24 times/min., every 20 min. The work was repeated six times (but in the hot room only after some days of acclimatization). The rectal temperature generally approached 102° F. after 2 hr. in the hot room, with a sweat loss of about 1 l./hr. after the first hour. The sweat loss was replaced every 20 min. to preserve water balance. Sixty-six experiments in the heat and twenty-five in the cold were made. The response to 750 c.c. of water in the cold without work was studied on sixty occasions. The results have been consistent. Fig. 1 shows typical findings on one subject.

In the cold, with or without work, diuresis varying in degree (cf. Barclay & Nutt, 1944) was obtained in nearly every case. Following extra water in the morning, the afternoon response was generally quite marked, about 700 c.c. urine being returned during the exercise period. After work, the average rate of urine flow was very low. *In the heat*, on sixty of the sixty-six occasions, the urinary output fell markedly in the 2 hr. work period, in spite of total replacement of sweat loss. Sometimes the fall was delayed until about the time sweating became profuse. The total excretion was nearly always less than half, often less than a third, of that in the cold. A diuresis usually occurred about an hour after leaving the hot room (Fig. 1).

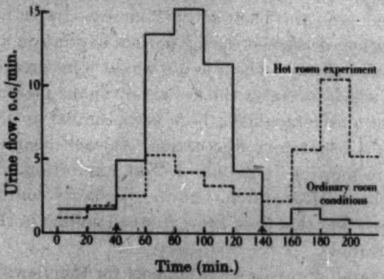

Fig. 1. Urinary flow before and after drinking 750 c.c. water. At first arrow 750 c.c. water drunk. Between arrows 5 min. work carried out every 20 min.

The cause of this consistent reduction in urine output in the heat cannot yet be stated. Clearly a failure in absorption of water from the alimentary tract must be distinguished from a specific alteration in kidney function.

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Vagal activity and the tachypnoea produced by multiple pulmonary emboli.

By E. G. WALSH and D. WHITTERIDGE. *University Laboratory of Physiology, Oxford*

One current hypothesis of the cause of tachypnoea in multiple pulmonary emboli is that the pulmonary stretch endings become hypersensitive (Partridge, 1935). Alternatively, Megibow, Katz & Feinstein (1943) have suggested that the vascular changes in the pulmonary circulation may stimulate the respiratory centre. We have re-investigated the electrical activity in the peripheral end of fibres cut from the side of the cervical vagus during the injection of suspensions of starch.

We have failed to find any consistent effect on the sensitivity of the vagal stretch endings. In cats and rabbits ventilated artificially, the mean change in the peak frequency of single stretch endings was −0·4 ± 4·2 impulses per sec. in nine experiments. In four animals which were breathing spontaneously there was no change in the sensitivity of the stretch endings, even though the respiratory rate in one case was doubled.

Of the fibres which show a cardiac rhythm, the depressor fibres showed no change until the systemic blood pressure began to drop at the end of the experiment. Other fibres were found which had groups of impulses corresponding to the *a*, *c* and *v* waves of the venous pulse, and which varied with the effective venous pressure. Their activity increased with normal inspiration and artificial deflation, decreased during normal expiration and was abolished by positive pressure inflation. The *a* and *v* waves were greatly increased after starch embolism, which increases the systemic venous pressure.

In another fibre, discharges have been seen, similar to those in depressor fibres, apart from a respiratory fluctuation. At each heart beat, there were 7–8 impulses at inspiration, but only 1–2 at expiration. Its systolic peak frequency rose a little after starch, but its discharge continued longer into diastole. We believe that this fibre arose from an ending in the neighbourhood of a pulmonary arteriole.

We think these results provide some support for the views of Megibow *et al.* (1943).

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